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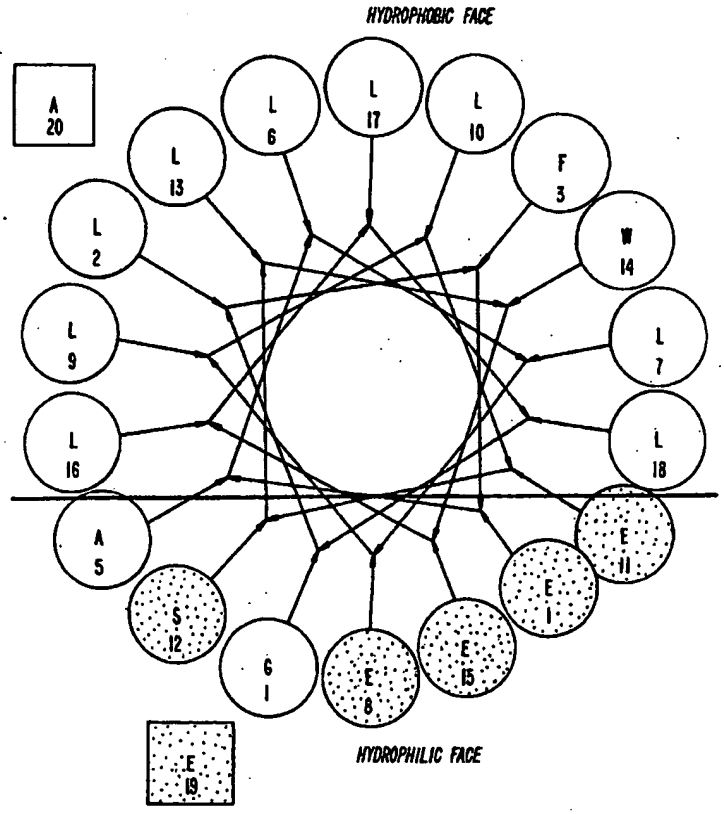
(54) Title: NUCLEIC ACID TRANSPORTERS FOR DELIVERY OF NUCLEIC ACIDS INTO A CELL

(57) Abstract

Nucleic acid transporter systems for delivery of nucleic acid to a cell. The nucleic acid transporter includes a binding complex. The binding complex contains a binding molecule which noncovalently binds to the nucleic acid. The binding complex can also contain a binding molecule which is associated with a surface ligand, nuclear ligand or a lysis agent. These may be associated with the binding molecule by spacers. In addition, the transporter may include a nucleic acid with a combination of the above binding complexes or binding molecules.



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DESCRIPTION

Nucleic Acid Transporters For Delivery of Nucleic Acids Into A Cell

Field of the Invention

The invention was partially supported by a grant from the United States government under grant number US-PH5 P01 HL50422 awarded by the National Institute of Health. The U.S. government may have rights in the invention.

Background of the Invention

This invention relates to gene therapy using a transporter system for delivering nucleic acid into a cell.

Recombinant retroviral vectors have been used for delivery of genes to cells of living animals. Morgan et al., *Annu. Rev. Biochem.*, 62:191-217 (1993). Retroviral vectors permanently integrate the transferred gene into the host chromosomal DNA. In addition to retroviruses, other virus have been used for gene delivery. Adenoviruses have been developed as a means for gene transfer into epithelial derived tissues. Stratford-Perricaudet et al., *Hum. Gene. Ther.*, 1:241-256 (1990); Gilardi et al., *FEBS*, 267:60-62 (1990); Rosenfeld et al., *Science*, 252:4341-4346 (1991); Morgan et al., *Annu. Rev. Biochem.*, 62:191-217 (1993). Recombinant adenoviral vectors have the advantage over retroviruses of being able to transduce nonproliferating cells, as well as an ability to produce purified high titer virus.

In addition to viral-mediated gene delivery, a more recent means for DNA delivery has been receptor-mediated endocytosis. Endocytosis is the process by which eucaryotic cells continually ingest segments of the plasma membrane in the form of small endocytotic vesicles. Alberts et al., *Mol. Biol. Cell*, Garland Publishing Co., New York, 1983. Extracellular fluid and material

dissolved in it becomes trapped in the vesicle and is ingested into the cell. *Id.* This process of bulk fluid-phase endocytosis can be visualized and quantified using a tracer such as enzyme peroxidase introduced into the extracellular fluid. *Id.* The rate of constitutive endocytosis varies from cell type to cell type.

Endocytotic vesicles form in a variety of sizes and shapes and are usually enlarged by fusing with each other and/or with other intracellular vesicles. Stryer, *Bioch.*, Freeman and Co., New York (1988). In most cells the great majority of endocytotic vesicles ultimately fuse with small vesicles called primary lysosomes to form secondary lysosomes which are specialized sites of intra-cellular digestion. *Id.* The lysosomes are acidic and contain a wide variety of degradative enzymes to digest the macromolecular contents of the vesicles. Silverstein et al., *Annu. Rev. Biochem.*, 46:669-722 (1977); Simianescu et al., *J. Cell Biol.*, 64:586-607 (1975).

Many of the endocytotic vesicles are clathrin-coated and are formed by invagination of coated regions of the plasma membrane called coated pits. Coated pits and vesicles provide a specialized pathway for taking up specific macromolecules from the extracellular fluid. This process is called receptor-mediated endocytosis. Goldstein et al., *Nature*, 279:679-685 (1979); Pearse et al., *Annu. Rev. Biochem.*, 50:85-101 (1981); Postan et al., *Annu. Rev. Physiol.*, 43:239-250 (1981). The macromolecules that bind to specific cell surface receptors are internalized via coated pits. Goldstein, *supra*. Receptor-mediated endocytosis is a selective mechanism enabling cells to ingest large amounts of specific ligands without taking in correspondingly large amounts of extracellular fluid. Goldstein, *supra*.

One such macromolecule is low density lipoprotein ("LDL"). Numerous studies have been performed involving LDL and the receptor-mediated endocytotic pathway. In addition to LDL, many other cell surface receptors have

been discovered to be associated with coated pits and receptor-mediated endocytosis. Pastan et al., *Annu. Rev. Physiol.*, 43:239-250 (1981). For example, studies have analyzed the hormone insulin binding to cell surface receptors and entering the cell via coated pits. Stryer et al., *Biochemistry*, Freeman & Co., New York (1988); Alberts et al., *Molecular Biology of the Cell*, Garland Publishing, New York (1983). In addition, it has been determined that some cell surface receptors associate with coated pits only after ligand binding. Pastan, *supra*.

Taking advantage of receptor-mediated endocytosis, the asialoglycoprotein receptor has been used in targeting DNA to HepG2 cells *in vitro* and liver cells *in vivo*. Wu et al., *J. Biol. Chem.*, 262:4429-4432 (1987); Wu et al., *Bio.*, 27:887-892 (1988); Wu et al., *J. Biol. Chem.*, 263:14620-14624 (1988); Wu et al., *J. Biol. Chem.*, 264:16985-16987 (1989); Wu et al., *J. Biol. Chem.*, 266:14338-14342 (1991). These studies used asialoorosomucoid covalently linked to polylysine with water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide or with 3'(2'pyridyldithio)propionic acid n-hydroxysuccinimide ester. Polylysine in the studies above bound DNA through ionic interaction. The DNA was ingested by endocytosis.

Other studies have utilized transferrin and the transferrin receptor for delivery of DNA to cells *in vitro*. Wagner et al., *P.N.A.S.*, 87:3410-3414 (1990). These studies modified transferrin by covalently coupling transferrin to polylysine. *Id.* The polylysine interacted ionically with DNA. Delivery of DNA occurred to cells through the transferrin receptor. Such analyses were performed *in vitro*. *Id.* Cotten et al., *P.N.A.S.*, 87:4033-4037 (1990); Zenk et al., *P.N.A.S.*, 87:3655-3659 (1990).

In addition to DNA, other macromolecules can also be delivered by receptor-ligand systems. Leamon et al., *P.N.A.S.*, 88:5572-5576 (1991); Leamon et al., *J. Biol.*

Chem., 267:24966-24971 (1992). In particular these studies have involved the folate receptor, an anchored glycosylphosphatidyl protein, which is excluded from coated pits and cycles in and out of the cells by caveolae. Anderson et al., *Science*, 252:410-411 (1992). This uptake mechanism has been called potocytosis. *Id.* Folate conjugated enzymes have been delivered into cells through this receptor system and retained activity for at least six hours. Leamon et al., *P.N.A.S.*, 88:5572-5576 (1991). Folate receptors have limited tissue distribution and are overexpressed in several malignant cell lines derived from many tissues. Weitman et al., *Cancer Res.*, 52:3396-3401 (1992); Weitman et al., *Cancer Res.*, 52:6708-6711 (1992); Campbell, *Cancer Res.*, 51:5329-5338 (1991); Coney, *Cancer Res.*, 51:6125-6123 (1991). Other studies have also used biotin or folate conjugated to proteins by biotinylation for protein delivery to the cell. Low et al., U.S. Patent 5,108,921.

Once DNA or macromolecules are targeted to a cell for delivery, the DNA or macromolecule must be released from the endosome to function as a therapeutic agent. If not, the delivery of DNA and macromolecule will be hindered by lysosomal degradation. Studies have analyzed the endosomal/lysosomal degradation process. It has been determined that organisms which are internalized via receptor-mediated endocytosis or receptor:ligand systems, like viruses and other microorganisms, escape lysosomal degradation in order to function. The entry mechanism of some viruses have been studied extensively. For some viruses outer membrane proteins have been demonstrated to be important for endosomal escape. Marsh et al., *Adv. Virus Res.*, 36:107-151 (1989). Other studies have focused on methods to prevent lysosomal degradation. These studies have used substances which pertubate endosomal/lysosomal function. Mellmann et al., *Ann. Rev. Biochem.*, 55:663-700 (1986). These substances have only been used *in vitro*.

In addition, studies show that the entire virus-shell is necessary for efficient endosomal lysis. Marsh et al., *Adv. Virus Res.*, 36:107-151 (1989). Studies have also demonstrated that adenovirus will enhance transferrin-polylysine mediated gene delivery. Curiel, *P.N.A.S.*, 88:8850-8854 (1991). These studies improved gene expression *in vitro* by using a replication defective adenovirus incorporated into DNA complexes. The effect of the adenovirus is to lyse the endosome before the contents can either be routed to the lysosome or recycled to the cell surface. To reduce virally induced cell death, adenovirus has been coupled enzymatically to polylysine through the ϵ-NH_2 of lysine and the γ-carboxyl of glutamic acid. Wagner et al., *P.N.A.S.*, 89:6099-03 (1992). Chemical coupling of polylysine with the acidic residues of adenovirus also accomplishes the same objective.

In addition to adenoviruses, peptide sequences from other viruses, such as influenza, have been used to achieve endosome rupture. Wagner et al., *P.N.A.S.*, 89:7934-7938 (1992). A lytic peptide from influenza hemagglutinin has been used to augment gene transfer by transferrin-polylysine-DNA complexes. *Id.* This virus-like genetic transfer vehicle has been shown to be functional *in vitro* but 100-fold less effective than adenovirus, based on the delivery and expression of the luciferase reporter construct. *Id.*

Other viruses have also been used for lysis purposes, like human immunodeficiency virus ("HIV"). U.S. Patent 5,149,782 (Chang et al., issued September 22, 1992). Peptide segments from HIV have been suggested to be useful as membrane blending agents to deliver nucleic acids. *Id.* These peptides are fusogenic and allow the associated nucleic acid or molecular conjugate to be inserted into the cellular plasma membrane. *Id.* These peptides are 10-30 amino acids in length and are hydrophobic. The fusion proteins used contain repetitious Phe-X-Gly sequences, where X is a nonpolar amino acid residue. *Id.*

A number of bacteria are also internalized via receptor-mediated endocytosis and are liberated from the endosome by production of toxins. These toxins lyse the endosomal membrane. Moulder, *Microbiol. Rev.*, 49:298-337 (1985). *Listeria monocytogenes* produce a membranolytic toxin called listeriolysin. Cossart et al., *Mol. Biol. Med.*, 6:463-474 (1989); Tilney et al., *J. Cell Bio.*, 109:1597-1608 (1989). Studies have shown that no other cofactors are needed for endosomal escape of *Listeria monocytogenes*. Bielecki et al., *Nature*, 345:175-176 (1990).

The listeriolysin toxin forms pores in membranes which contain cholesterol. These pores are large enough for macromolecules like immunoglobulins to pass. Ahnert-Hilger et al., *Mol. Cell Biol.*, 31:63-90 (1989); Geoffroy et al., *J. Bacteriol.*, 172:7301-7305 (1990).

In addition, numerous studies have analyzed the role of polyamines in the intracellular processes involving nucleic acids. In particular, studies show that polyamines enhance both transcription and translation, and are involved in maintaining tRNA structure and activity. Tabor, et al., *Annu. Rev. Biochem.*, 171:15-42 (1970); Cohen, *Nature*, 274:209-210 (1978). Furthermore, polyamines have been shown to condense nucleic acids which may be utilized in the cell for packaging processes. Gosule, et al., *J. Mol. Biol.*, 121:311-326 (1978); Chattoraj, et al., *J. Mol. Biol.*, 121:327-337 (1978); Riemer, et al., *Biopolymers*, 17:785-794 (1970). Additional studies have found polyamines are active in ribosome stabilization and in the packaging of DNA into phage heads. Stevens, et al., *Ann. N.Y. Acad. Sci.*, 171:827-837 (1970); Wilson, et al., *Biochemistry*, 18:2192-2196 (1979).

The above studies have investigated the electrostatic component in the interaction of the polyamines, such as large poly-L-lysine molecules, with nucleic acids. The average chain length of these poly-L-lysines ranged from

50-200. Use of the poly-L-lysines, however, have been shown to be toxic to cells in nM concentrations thereby limiting their applicability. Such studies have also been performed with spermine, spermidine and putrescine. Braunlin, et al., *Biopolymers*, 21:1301-1314 (1982).

Summary of the Invention

Applicant has determined that it is useful to construct nucleic acid transporter systems for enhanced delivery of nucleic acid into the cell. These particular transporter systems enhance delivery of nucleic acid into the cell by using synthetic lysis and nucleic acid binding molecules. In particular, the specific lysis agents are useful in disrupting the endosome thereby allowing the nucleic acid to avoid lysosomal degradation. The specific binding molecules are useful in delivering to the cell stabilized and condensed nucleic acid. In addition, these specific binding molecules are useful in delivering stabilized and condensed nucleic acid into the nucleus of the cell. These transporters can be used to treat diseases by enhancing delivery of specific nucleic acid to the appropriately targeted cells. These transporters can also be used to create transformed cells, as well as transgenic animals for assessing human disease in an animal model.

The present invention takes advantage of lysis agents to avoid the problems of endosomal/lysosomal degradation in the delivery of nucleic acid to a cell. In particular, the present invention features use of a nucleic acid transporter system with nucleic acid binding complexes that includes a specific lysis agent capable of releasing nucleic acid into the cellular interior from the endosome. The nucleic acid can be efficiently released without endosomal/lysosomal degradation. Once released into the cellular interior, the binding complexes help target the nucleic acid to the nucleus.

The present invention also takes advantage of DNA binding molecules in order to increase DNA stability and DNA delivery to cells. In particular, the present invention features use of nucleic acid transporters with nucleic acid noncovalently bound to peptides capable of condensing the nucleic acid. These binding molecules provide smaller, or condensed, and more stable nucleic acid particles for delivery, thereby enhancing the transfection rates of the nucleic acid into the cell and into the nucleus.

By taking advantage of the characteristics of both the lysis agents and binding molecules, the present invention enhances delivery of nucleic acid by the nucleic acid transporter system. These components can be used alone, together or with other components of the nucleic acid transporter described below and disclosed in PCT publication WO 93/18759, Woo et al., entitled "A DNA Transporter System and Method of Use," the whole of which (including drawings) is hereby incorporated by reference. The transporter system, together with the lysis and binding molecule, enhances the delivery of nucleic acid to specific cells by enhancing the release of stable, condensed nucleic acid from the endosome into the cellular interior.

In addition to the nucleic acid binding molecule and the nucleic acid binding complex containing the lysis agent, the present invention also features various nucleic acid binding complexes which contain a surface ligand and a nuclear ligand as well. The surface ligands are capable of binding to a cell surface receptor and entering a cell through cytosis (*e.g.*, endocytosis, potocytosis, pinocytosis). By using surface ligands specific to certain cells, nucleic acid can be delivered using the nucleic acid transporter systems directly to the desired tissue. The nuclear ligands are capable of recognizing and transporting nucleic acid through the nuclear membrane to the nucleus of a cell. Such nuclear ligands help enhance the

binding molecules' ability to target nucleic acid to the nucleus.

The abilities of the above transporters to deliver nucleic acid to specific cells and to the nucleus also allows transgenic animal models to be used for the dissection of molecular carcinogenesis and disease, assessing potential chemical and physical carcinogens and tumor promoters, exploring model therapeutic avenues as well as livestock agricultural purposes. Furthermore, the above nucleic acid transporter system advantages allow methods for administration and treatment of various diseases. In addition, the above nucleic acid transporter systems can be used to transform cells to produce particular proteins, polypeptides, and/or RNA. Likewise, the above nucleic acid transporter systems can be used *in vitro* with tissue culture cells. *In vitro* uses allow the role of various nucleic acids to be studied by targeting specific expression into specifically targeted tissue culture cells.

A first aspect of the present invention features a nucleic acid transporter system for delivering nucleic acid into a cell. The nucleic acid transporter includes a nucleic acid binding complex containing a binding molecule noncovalently bound to nucleic acid and associated with a lysis agent. In addition, the transporter can also include an additional binding molecule noncovalently bound to the nucleic acid. The nucleic acid binding complex and/or the additional binding molecule may be noncovalently bound to the nucleic acid at the same time, *i.e.*, simultaneously, and in various proportions. Furthermore, the lysis agent can be associated with the respective binding molecule by a spacer.

The term "lysis agent" as used herein refers to a molecule, compound, protein or peptide which is capable of breaking down an endosomal membrane and freeing the contents into the cytoplasm of the cell. The lysis agent

can work by: (1) a membrane fusion mechanism, *i.e.*, fusogenic, whereby the lysis agent associates or fuses with the cell membrane to allow the endosomal contents to leak into the cytoplasm; (2) a membrane destabilization mechanism whereby the lysis agent disrupts the structural organization of the cell membrane thereby causing leakage through the endosome into the cytoplasm; or (3) other known or unknown mechanisms which cause endosomal lysis. This term includes, but is not limited to, synthetic compounds such as the JTS-1 peptide, viruses, lytic peptides, or derivatives thereof. The term "lytic peptide" refers to a chemical grouping which penetrates a membrane such that the structural organization and integrity of the membrane is lost. As a result of the presence of the lysis agent, the membrane undergoes lysis, fusion or both.

In the present invention, a preferred lysis agent is the JTS-1 peptide or derivatives thereof. The amino acid sequence of JTS-1 lytic peptide is GLFEALLELLESLWELLLEA. One skilled in the art will readily appreciate and understand that such nomenclature is the standard notation accepted in the art for designating amino acids. The JTS-1 lytic peptide and derivatives are designed as an α-helix, which contains a sequence of amino acids such that the side chains are distributed to yield a peptide with hydrophobic and hydrophilic sides. Such α-helixes are termed amphipathic or amphiphilic. The hydrophobic side contains highly apolar amino acid side chains, both neutral and non-neutral. The hydrophilic side contains an extensive number of glutamic acids but could also contain aspartic acid, as well as polar or basic amino acids. The JTS-1 peptide would include any derivatives or modifications of the backbone thereof. The lytic peptide undergoes secondary structure changes at acidic pH resulting in the formation of oligomeric aggregates which possess selective lytic properties.

In general, parameters that are important for amphiphilic peptide lysis activity include the following.

First, Hydrophobicity: The peptide must have a high enough hydrophobicity of the hydrophobic face to interact with and penetrate phospholipid-cholesterol membranes, *i.e.*, lipid binding per se is not sufficient. Red cell hemolysis assays give better indications of which peptides will have useful activity. Second, Peptide aggregation: The ability to aggregate plays an important role in lysis and transfection. Third, pH sensitivity: The amphiphilic peptide must be pH sensitive. Lysis activity can be controlled by the introduction of lysine, arginine and histidine residues into the hydrophilic face of JTS-1. Fourth, Lipid membrane interaction: The peptide must have a hydrophobic carboxyl terminal to permit interaction with lipid membranes, *e.g.*, tyrosine substitution for tryptophan greatly reduces activity. Finally, Peptide chain length: The length must be greater than twelve residues in order to get stable helix formation and lipid membrane penetration and rupture.

The term "derivative" as used herein refers to a peptide or compound produced or modified from another peptide or compound of a similar structure. This could be produced in one or more steps. The term "modified" or "modification" as used herein refers to a change in the structure of the compound or molecule. However, the activity of the derivative, modified compound or molecule is retained, enhanced, increased or similar to the activity of the parent compound or molecule. This would include the change of one amino acid in the sequence of the peptide or the introduction of one or more non-naturally occurring amino acids or other compounds. This includes a change in a chemical body, a change in a hydrogen placement, or any type of chemical variation. In addition, "analog" as used herein refers to a compound that resembles another structure, *e.g.*, JTS-1, K_8, K_N, or spermine (discussed below). Analog is not necessarily an isomer. The above are only examples and are nonlimiting.

For example, the JTS-1 peptide can be modified to change the L in position 2 to an F so as to have the structure GFFEALLELLESLWELLLEA. Such a change can increase the hydrophobicity of the peptide. Furthermore, increasing the length of the peptide would also be a modification, *i.e.*, GLFEALLELLESWELLLGLFEA. Such a change can enhance the efficiency of the JTS-1 peptide. A change in the S at position 12 to a K to modify JTS-1 to GLFEALLELLEKLWELLLEA can shift the pH optimum for lysis and enhance proteolysis. The above are only examples and meant to be nonlimiting.

Other useful lysis agents include, but are not limited to, peptides of the Othromyxoviridae, Alphaviridae and Arenaviridae. Lysis agents also can include *Pep*24, *Pep*25, *Pep*26, (see PCT publication WO 93/18759, hereby incorporated by reference, including drawings), any appropriate bacteria toxin, bacteria, adenovirus, para-influenza virus, herpes virus, retrovirus, hepatitis virus, or any appropriate lytic peptide or protein from a virus or bacteria. This includes use of any subfragments of the above which will provide endosomal escape activity. Particular bacterial toxins may include cytolytic toxins or active fragments from alveolysin, bifermentolysin, botulinolysin, capriciolysin, cereolysin O, chauveolysin, histolyticolysin O, ivanolysin, laterosporolysin, oedematolysin O, listeriolysin O, perfringolysin O, pneumolysin, sealigerolysin, septicolysin O, sordellilysin, streptolysin O, tetanolysin or thuringolysin O.

In addition to JTS-1 in another preferred embodiment the lysis agent can be a replication deficient virus. As used herein, the term "replication deficient" refers to a virus lacking one or more of the necessary elements for replication. In one preferred embodiment, the lysis agent can also be the adenovirus of the structure F, *Pep*24, *Pep*25, or *Pep*26. In still another embodiment, bacteria toxins, listeriolysin or perfringolysin can be used. All

of the above are disclosed in PCT publication WO 93/18759, which is hereby incorporated by reference, including drawings. The above are only examples and are nonlimiting.

Lysis agents as used herein are pH sensitive. The pH optimum is determined by the sequence and the content of acidic and basic amino acid side chains. After cointernalization of the nucleic acid complex containing the lysis agent throughout the same coated pit on the plasma membrane of the cell, the decrease in pH that occurs immediately after endosome formation causes spontaneous lysis of the endosome. The nucleic acid is then released into the cytoplasm. The above is a nonlimiting example.

The term "binding molecule" as used herein refers to a molecule, compound, protein or peptide which is capable of stabilizing and condensing nucleic acid. This will include, but is not limited to, components which are capable of stabilizing and condensing nucleic acid by electrostatic binding, hydrophobic binding, hydrogen binding, intercalation or forming helical structures with the nucleic acid, including interaction in the major and/or minor grove of DNA. The term binding molecule can also be referred herein as condensing agent. The binding molecule is capable of noncovalently binding to nucleic acid. One skilled in the art will readily appreciate the meaning of noncovalent. The binding molecule is also capable of associating with a surface ligand, a nuclear ligand, and/or a lysis agent.

The term "associated with" as used herein refers to binding, attaching, connecting or linking molecules through covalent means or noncovalent means. One skilled in the art will readily appreciate the meaning of covalent and noncovalent. "Associated with" includes, but is not limited to, a binding molecule associated with a surface ligand, nuclear ligand and/or a lysis agent. In addition,

it includes the association of a spacer (discussed below) with the above components.

In the present invention, a preferred binding molecule is the peptide K_8. The amino acid sequence of K_8 is YKAKKKKKKKKWK. In another preferred embodiment, the binding molecule is any peptide with the formula $YKAK_NWK$, where N can be between 1-40. This formula or amino acid structure can be referred to as "K_N". This would include use of any subfragments of the above which provide nucleic acid stability and condensing characteristics. Furthermore, this would include any derivatives, analogs or modifications of K_8 or the general $YKAK_NWK$ structure K_N.

The above peptides can include lysine or arginine residues for electrostatic binding to nucleic acid. These positively charged amino acids help hold the nucleic acid intact. The binding molecule can also contain tyrosine which is useful in determining peptide concentration and iodination for tracking purposes *in vitro* and *in vivo*. Tryptophan also increases the stability of interaction with the nucleic acid through intercalation. In addition, binding of the peptide to DNA quenches tryptophan fluorescence and allows the kinetics and thermodynamics of complex formation to be determined. The binding molecule can also contain helix forming residues such as tryptophan, alanine, leucine or glutamine. These can act as spacers which allow the cationic residues to adopt an optimal configuration for interaction with the nucleic acid in a helical manner, resulting in a more stable complex. Furthermore, the binding molecule can also include a stabilized cyclic version of K_8 or the general $YKAK_NWK$ structure K_N. Such a cyclic version can be formed by introducing a lactam or disulfide bridge. Likewise, dimers of K_8 or K_N can also be used as a binding molecule.

In general, parameters that are important for binding molecules include the following. First, the peptide must contain sufficient lysine or arginine residues to permit ionic interaction with the DNA. Second, the peptide must

have sufficient length to form a stable helix, eleven or twelve residues, and condense the DNA to small particles, *e.g.*, K_4 forms larger particles than K_8. Third, the peptide helix that forms upon interaction with DNA can be stabilized by leucine zipper formation which gives a condensing agent less susceptible to ionic strength. Finally, the lysine or arginine sequence of the condensing peptide serves as an additional function as a nuclear localization sequence.

The binding molecule can also include, but is not limited to, spermine, spermine derivative, spermidine, histones, polylysine, polyamines and cationic peptides. As with K_8 or K_N, this includes, but is not limited to, analogs, modifications or derivatives of the above compounds.

Spermine derivatives include compounds D, IV, VII, XXI, XXXIII, XXXVI, LIV, LVI, LXXXII, LXXXIV and CX as described in PCT publication WO 93/18759, hereby incorporated by reference, including drawings. When used with the nucleic acid transporter system, the binding molecules, such as K_8, K_N or spermine, whether associated with a surface ligand, nuclear ligand, lysis agent, or separate therefrom, can be different or similar binding molecules and bound at the same time, *i.e.*, simultaneously and in various proportions. In a preferred embodiment the binding molecule is a spermine derivative D, as shown in the above referenced publication.

K_8, K_N, and spermine have advantages over poly-L-lysine as used for the binding molecule. For example, the binding properties of K_8, K_N, or spermine have advantages over the binding properties of poly-L-lysine. First, the intranuclear K_8, K_N, or spermine concentration is approximately 3 to 10 mmol. This is higher than studies with poly-L-lysine, which suggest more efficient transfer of nucleic acid to the nucleus. Second, the spacing of the amino groups of K_8, K_N or spermine is such that this naturally occurring polycation fits into the major groove

of the DNA double helix with an exact fit. While the polycationic poly-L-lysine interacts electrostatically with the phosphates in the groove of DNA, the fit is not as precise. Finally, the theoretical association/dissociation kinetics of the DNA/K_8, K_N or spermine interaction are more rapid than for DNA/poly-L-lysine interactions. This is advantageous in the K_8, K_N or spermine/DNA mix for the release of the DNA inside the cell.

The term "nucleic acid transporter system" as used herein refers to a molecular complex which is capable of efficiently transporting nucleic acid through the cell membrane. This molecular complex is bound to nucleic acid noncovalently. In addition to nucleic acid, other macromolecules, including but not limited to, proteins, lipids and carbohydrates can also be delivered using the transporter system. The nucleic acid transporter system is capable of transporting nucleic acid in a stable and condensed state. It is also capable of releasing the noncovalently bound nucleic acid into the cellular interior. Furthermore, the nucleic acid transporter prevents degradation of the nucleic acid by endosomal lysis. In addition, although not necessary, the nucleic acid transporter system can also efficiently transport the nucleic acid through the nuclear membrane, as discussed below.

The nucleic acid transporter system as described herein can contain, but is not limited to, six components. It comprises, consists or consists essentially of: (1) a nucleic acid or other macromolecule with a known primary sequence that contains the genetic information of interest or a known chemical composition; (2) an agent capable of stabilizing and condensing the nucleic acid or macromolecule in (1) above; (3) a lysis moiety that enables the transport of the entire complex from the cell surface directly into the cytoplasm of the cell; (4) a moiety that recognizes and binds to a cell surface

receptor or antigen or is capable of entering a cell through cytosis; (5) a moiety that is capable of moving or initiating movement through a nuclear membrane; and/or (6) a nucleic acid or macromolecular molecule binding moiety capable of covalently binding the moieties of (3), (4) and (5), above. The term "consisting of" is used herein as it is recognized in the art. The transporter "consisting essentially of" the six moieties above includes variation of the above moieties. Such a variation may make use of less than all six of the moieties listed above. This is only an example and is nonlimiting.

The term "delivery" refers to transportation of a molecule to a desired cell or any cell. Delivery can be to the cell surface, cell membrane, cell endosome, within the cell membrane, nucleus or within the nucleus, or any other desired area of the cell. Delivery includes not only transporting nucleic acid but also other macromolecules including, but not limited to, proteins, lipids, carbohydrates and various other molecules.

The term "nucleic acid" as used herein refers to DNA or RNA. This would include naked DNA, a nucleic acid cassette, naked RNA, or nucleic acid contained in vectors or viruses. These are only examples and are not meant to be limiting. The term "expression" includes the efficient transcription by the cell of the transported gene or nucleic acid. Expression products may be proteins, polypeptides or RNA. In addition, the nucleic acid can be antisense RNA, oligonucleotides or ribozymes as well.

A variety of proteins and polypeptides can be encoded by the nucleic acid. Those proteins or polypeptides which can be expressed include hormones, growth factors, enzymes, clotting factors, apolipoproteins, receptors, drugs, oncogenes, tumor antigens, tumor suppressors, cytokines, viral antigens, parasitic antigens and bacterial antigens. Specific examples of these compounds include proinsulin, insulin, growth hormone, androgen receptors, insulin-like growth factor I, insulin-like

growth factor II, insulin growth factor binding proteins, epidermal growth factor, TGF-α, TGF-β, dermal growth factor (PDGF), angiogenesis factors (acidic fibroblast growth factor, basic fibroblast growth factor and angiogenin), matrix proteins (Type IV collagen, Type VII collagen, laminin), oncogenes (*ras*, *fos*, *myc*, *erb*, *src*, *sis*, *jun*), E6 or E7 transforming sequence, p53 protein, cytokine receptor, IL-1, IL-6, IL-8, IL-2, α, β, or γIFN, GMCSF, GCSF, viral capsid protein, and proteins from viral, bacterial and parasitic organisms. Other specific proteins or polypeptides which can be expressed include: phenylalanine hydroxylase, α-1-antitrypsin, cholesterol-7α-hydroxylase, truncated apolipoprotein B, lipoprotein lipase, apolipoprotein E, apolipoprotein A1, LDL receptor, molecular variants of each, and combinations thereof. One skilled in the art readily appreciates that these proteins belong to a wide variety of classes of proteins, and that other proteins within these classes can also be used. These are only examples and are not meant to be limiting in any way.

It should also be noted that the genetic material which is incorporated into the cells from the above nucleic acid transporter system includes (1) nucleic acid not normally found in the cells; (2) nucleic acid which is normally found in the cells but not expressed at physiological significant levels; (3) nucleic acid normally found in the cells and normally expressed at physiological desired levels; (4) other nucleic acid which can be modified for expression in cells; and (5) any combination of the above.

The term "nucleic acid binding complex" as used herein refers to a complex which includes a binding molecule. The binding molecule, as defined above, is capable of noncovalently binding to nucleic acid. The binding molecule is also capable of associating with a surface ligand, a nuclear ligand and/or a lysis agent. Furthermore, the binding complex can include a spacer

which associates with the surface, nuclear or lysis agent to the binding molecule. Spacers are defined in more detail below.

A second aspect of the present invention features a nucleic acid transporter system for delivery of a nucleic acid to a cell which includes a first nucleic acid binding complex containing a binding molecule noncovalently bound to nucleic acid and associated with a surface ligand. The transporter also includes a second nucleic acid binding complex containing a binding molecule noncovalently bound to nucleic acid and associated with a lysis agent. In addition, the transporter can also include an additional binding molecule noncovalently bound to the nucleic acid.

The binding complexes and/or binding molecules above can be noncovalently bound to the nucleic acid at the same time, *i.e.*, simultaneously, and in various proportions. As described above, the binding molecules can be the same or different molecules. Furthermore, the surface ligand or lysis agent can be directly associated with the binding molecules or associated by a spacer, as defined below.

The term "surface ligand" as used herein refers to a chemical compound or structure which will bind to a surface receptor of a cell. The term "cell surface receptor" as used herein refers to a specific chemical grouping on the surface of a cell for which the ligand can attach. Cell surface receptors can be specific for a particular cell, *i.e.*, found predominantly in one cell rather than in another type of cell (*e.g.*, LDL and asialoglycoprotein receptors are specific for hepatocytes). The receptor facilitates the internalization of the ligand and attached molecules. A cell surface receptor includes, but is not limited to, a folate receptor, biotin receptor, lipoic acid receptor, low-density lipoprotein receptor, asialoglycoprotein receptor, insulin-like growth factor type II/cation-independent mannose-6-phosphate receptor, calcitonin gene-related peptide receptor, insulin-like growth factor I receptor, nicotinic acetylcholine

receptor, hepatocyte growth factor receptor, endothelin receptor, bile acid receptor, bone morphogenetic protein receptor, cartilage induction factor receptor or glycosyl-phosphatidylinositol (GPI)-anchored proteins (*e.g.*, β-andrenargic receptor, T-cell activating protein, Thy-1 protein, GPI-anchored 5' nucleotidase). These are nonlimiting examples.

A receptor is a molecule to which a ligand binds specifically and with relatively high affinity. It is usually a protein or a glycoprotein, but may also be a glycolipid, a lipidpolysaccharide, a glycosaminoglycan or a glycocalyx. For purposes of this invention, epitopes to which an antibody or its fragments binds is construed as a receptor since the antigen:antibody complex undergoes endocytosis. Furthermore, surface ligand includes anything which is capable of entering the cell through cytosis (*e.g.*, endocytosis, potocytosis, pinocytosis).

As used herein, the term "ligand" refers to a chemical compound or structure which will bind to a receptor. This includes but is not limited to ligands such as asialoorosomucoid, asialoglycoprotein, folate, lipoic acid, biotin, as well as those compounds listed in PCT publication WO 93/18759, hereby incorporated by reference.

One skilled in the art will readily recognize that the ligand chosen will depend on which receptor is being bound. Since different types of cells have different receptors, this provides a method of targeting nucleic acid to specific cell types, depending on which cell surface ligand is used. Thus, the preferred cell surface ligand may depend on the targeted cell type.

A third aspect of the present invention features a nucleic acid transporter system for delivery of a nucleic acid into a cell which includes a first nucleic acid binding complex containing a binding molecule noncovalently bound to nucleic acid and associated with a surface ligand. The transporter also includes a second nucleic acid binding complex containing a binding molecule

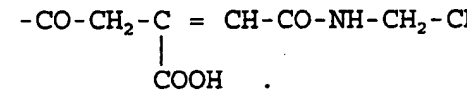
noncovalently bound to nucleic acid and associated with a nuclear ligand. The transporter also includes a third nucleic acid binding complex containing a binding molecule noncovalently bound to a nucleic acid and associated with a lysis agent. In addition, the transporter can include a fourth binding molecule noncovalently bound to said nucleic acid.

The nucleic acid binding complexes and/or binding molecules can be noncovalently bound to the nucleic acid at the same time, *i.e.*, simultaneously, and in various proportions. The binding molecules can be the same molecule or a combination of a different molecule as discussed above. Furthermore, the surface ligand, nuclear ligand, and lysis agent can be directly associated with the binding molecule or associated by a spacer as defined below.

The term "nuclear ligand" as used herein refers to a ligand which will bind a nuclear receptor. The term "nuclear receptor" as used herein refers to a chemical grouping on the nuclear membrane which will bind a specific ligand and help transport the ligand through the nuclear membrane. Nuclear receptors can be, but are not limited to, those receptors which bind nuclear localization sequences. Nonlimiting examples of nuclear ligands include those disclosed in PCT publication WO 93/18759, hereby incorporated by reference.

As noted above, the surface ligand, the nuclear ligand and/or the lysis agent can be associated directly to the binding molecule or can be associated with the binding molecule via a spacer. The term "spacer" as used herein refers to a chemical structure which links two molecules to each other. The spacer normally binds each molecule on a different part of the spacer molecule. The spacer can be hydrophilic molecules comprised of about 6 to 30 carbon atoms. The spacer can also contain between 6 to 16 carbon atoms. The spacer can include, but is not limited to, a hydrophilic polymer of $[(gly)_i(ser)_j]_k$ wherein i ranges from

1 to 6, j ranges from 1 to 6, and k ranges from 3 to 20. In addition, the spacer and binding molecule compounds include, but are not limited to, those compounds disclosed in PCT publication WO 93/18759, hereby incorporated by reference. Furthermore, the spacer may include, but is not limited to, repeating omega-amino acid of the structure $[NH-(CH_2CH_2)_n-CO-]_m$, where n = 1-3 and m = 1-20, a disulfide structure $(CH_2CH_2-S-S-CH_2CH_2-)_n$, where n = 1-20, or an acid sensitive bifunctional molecule with the structure



In one preferred embodiment of the present invention, the lysis agent is JTS-1, or derivative thereof, and the binding molecule K_8, or derivative thereof. Still another embodiment of the present invention can include a surface, nuclear ligand and the lysis agent as disclosed herein, and a binding molecule of K_8, K_N or derivative thereof. In still another embodiment, the surface and nuclear ligand can be one of those disclosed herein, the lysis agent can be JTS-1 or derivative thereof, and the binding molecule can be K_8, K_N or derivative thereof. These embodiments can also include the use of spacers as described above.

In one preferred embodiment of the above aspects, folate is used as the surface ligand and JTS-1 is used as the lysis agent. This transporter, as well as the other nucleic acid transporters described in this invention, can deliver to the cytosol other macromolecules besides nucleic acid including, but not limited to, proteins, lipids and carbohydrates. The binding complexes of this aspect can be noncovalently bound to the nucleic acid at the same time, *i.e.*, simultaneously, and in various proportions. The binding molecules can be the same or different and may attach to the ligands or lysis agents directly or by spacers as described above.

In addition to the above embodiment, a nucleic acid binding molecule, K_8, K_N or derivative, can also be used in

conjunction with either embodiment. The binding molecule can be noncovalently bound to the nucleic acid. More than one binding molecule can be noncovalently bound to the nucleic acid at the same time, *i.e.*, simultaneously, and in various proportions.

In another preferred embodiment, an asialoglycoprotein can be used as the surface agent, K_8, K_N or derivative as the binding molecule and JTS-1 or derivative, listeriolysin or perfringolysin as the lysis agent. Listeriolysin, perfringolysin or only a part of the toxins harboring the active subfragments need be used. Similarly, all microbial toxins and their active subfragments can be incorporated into the transporters of the present invention for endosomal escape.

A fourth aspect of the present invention features the JTS-1 composition and derivative. As discussed above, these compositions are advantageous in that they have endosomal lysis properties. When used with the nucleic acid transporter system as described above, JTS-1 or derivatives enhance the expression of nucleic acid targeted to a cell. The JTS-1 compound and derivatives are described below in more detail.

A fifth aspect of the present invention features the K_8 or K_N compositions and derivatives. As discussed above, these binding molecules are advantageous in that they have nucleic acid condensing/stabilizing properties. When used with the nucleic acid transporter system as described above, K_8 or K_N compositions and derivatives enhance the expression of nucleic acid targeted to a cell. The K_8 or K_N compounds and derivatives are described below in more detail.

In addition, as noted above, all the above aspects can feature a nucleic acid transporter system described above containing a plurality of nucleic acid binding complexes with a binding molecule noncovalently bound to nucleic acid and attached to a surface ligand, a nuclear ligand or a lysis agent. There may also be a plurality of

additional binding molecules separate from the binding complexes above. Spacers can be used to connect the surface ligand, nuclear ligand and/or lysis agent.

A sixth related aspect of the present invention features a method of using the above described nucleic acid transporters for delivery of a nucleic acid or a molecule to a cell. Such use includes both *in vivo* and *in vitro* uses. This would include cells transformed with the nucleic acid transporter system as described above for expression of nucleic acid targeted to the cell. As defined above, the nucleic acid may include nucleic acid containing genetic material and coding for a variety of proteins, polypeptides or RNA.

As used herein "transformation" or "transformed" is a mechanism of gene transfer which involves the uptake of nucleic acid by a cell or organism. It is a process or mechanism of inducing transient or permanent changes in the characteristics (expressed phenotype) of a cell. Such changes are by a mechanism of gene transfer whereby DNA or RNA is introduced into a cell in a form where it expresses a specific gene product or alters the expression or effect of endogenous gene products. Following entry into the cell, the transforming nucleic acid may recombine with that of the host. Such transformation is considered stable transformation in that the introduction of gene(s) into the chromosome of the targeted cell where it integrates and becomes a permanent component of the genetic material in that cell. Gene expression after stable transformation can permanently alter the characteristics of the cell leading to stable transformation. In addition, the transforming nucleic acid may exist independently as a plasmid or a temperate phage, or by episomes. An episomal transformation is a variant of stable transformation in which the introduced gene is not incorporated in the host cell chromosomes but rather remains in a transcriptionally active state as an extrachromosomal element.

Transformation can be performed by *in vivo* techniques as described below, or by *ex vivo* techniques in which cells are cotransfected with a nucleic acid transporter system containing nucleic acid and also containing a selectable marker. This selectable marker is used to select those cells which have become transformed. It is well known to those skilled in the art the type of selectable markers to be used with transformation studies.

The transformed cells can produce a variety of compounds selected from proteins, polypeptides or RNA, including hormones, growth factors, enzymes, clotting factors, apolipoproteins, receptors, drugs, tumor antigens, viral antigens, parasitic antigens, and bacterial antigens. Other examples can be found above in the discussion of nucleic acid. The product expressed by the transformed cell depends on the nucleic acid used. The above are only examples and are not meant to be limiting.

These methods of use would include the steps of contacting a cell with a nucleic acid transporter system as described above for a sufficient time to transform the cell. Cell types of interest can include, but are not limited to, liver, muscle, lung, endothelium, bone, blood, joints and skin.

The methods of use would also include a transgenic animal whose cells contain the nucleic acid referenced above delivered via the nucleic acid transporter system. These cells include germ or somatic cells. Transgenic animal models can be used for dissection of molecular carcinogenesis and disease, assessing potential chemical and physical carcinogens and tumor promoters, exploring model therapeutic avenues and livestock agricultural purposes.

The methods of use also include a method of treating humans, which is another aspect of the present invention. The method of treatment includes the steps of administering the nucleic acid transporters as described above so as to deliver a desired nucleic acid to a cell or

tissue for the purposes of expression of the nucleic acid by the cell or tissue. Cell or tissue types of interest can include, but are not limited to, liver, muscle, lung, endothelium, joints, skin, bone and blood.

The methods of treatment or use include methods for delivering nucleic acid into a hepatocyte by contacting a hepatocyte with the above referenced nucleic acid transporters. The surface ligand used with the nucleic acid transporter is one specific for recognition by hepatocyte receptors. In particular, the asialoorosomucoid protein is used as a cell surface ligand, K_8, K_N or a derivative as a binding molecule and JTS-1 or a derivative as a lysis agent. Furthermore, these methods of use also include delivery of nucleic acids using a transporter with JTS-1 and K_8 and no surface or nuclear ligands. The term "hepatocyte" as used herein refers to cells of the liver.

An aspect of the methods of treatment or use includes a method for delivering nucleic acid to muscle cells by contacting the muscle cell with one of the above referenced nucleic acid transporter system. The surface ligand used is specific for receptors contained on the muscle cell. In particular, the surface ligand can be insulin-like growth factor-I. In addition, the binding molecule can be a K_8, K_N or a derivative and the lysis agent can be JTS-1 or a derivative. Furthermore, these methods of treatment or use also include delivery of nucleic acids using a transporter with JTS-1 and K_8 and no surface or nuclear ligands. The term "muscle cell" as used herein refers to cells associated with striated muscle, smooth muscle or cardiac muscle.

Another aspect of the methods of treatment or use includes a method for delivering nucleic acid to bone-forming cells by contacting the bone-forming cell with the above referenced nucleic acid transporter system. The surface ligand used with the nucleic acid transporter system is specific for receptors associated with bone-

forming cells. In particular, the surface ligands can include, but are not limited to, bone morphogenetic protein or cartilage induction factor. In addition, the binding molecule of the nucleic acid transporter can be K_8, K_N or a derivative, and the lysis agent JTS-1 or a derivative thereof. Furthermore, these methods of treatment or use also include delivery of nucleic acids using a transporter with JTS-1 and K_8 and no surface or nuclear ligands. As used herein the term "bone-forming cell" refers to those cells which promote bone growth. Nonlimiting examples include osteoblasts, stromal cells, inducible osteoprogenitor cells, determined osteoprogenitor cells, chondrocytes, as well as other cells capable of aiding bone formation.

Another related aspect of the methods of treatment or use includes a method for delivering nucleic acid to a cell using the above referenced nucleic acid transporter system. The nucleic acid transporter system uses folate as a ligand. In addition, the nucleic acid transporter can use JTS-1 or a derivative as a lysis agent, and K_8, K_N or a derivative thereof as a binding molecule. This method targets cells which contain folate receptors, including, but not limited to, hepatocytes.

Still another related aspect of the methods of treatment or use includes a method for delivering nucleic acid to synovialcytes or macrophages using the above referenced nucleic acid transporter system. The nucleic acid transporter system uses a ligand recognized by synovialcytes and/or macrophages. In addition, the nucleic acid transporter can use JTS-1 or a derivative as a lysis agent, and K_8, K_N or a derivative thereof as a binding molecule. Furthermore, this method of use also includes delivery of nucleic acids using a transporter with JTS-1 and K_8 and no surface or nuclear ligands. The term "synovialcytes" refers to cells associated with the joints or with the fluid space of the joints.

In addition to the above methods, the method of use also includes delivery using a nuclear ligand binding complex as well. Such nuclear transporters would help direct the nucleic acid to the nucleus. Furthermore, the above methods of use also include nucleic acid transporters with the binding molecule and the lysis agent, or a plurality thereof.

The nucleic acid transporters of the above methods may be administered by various routes. The term "administration" or "administering" refers to the route of introduction of the nucleic acid transporter or carrier of the transporter into the body. Administration may be intravenous, intramuscular, topical, olfactory or oral. Administration can be directly to a target tissue or through systemic delivery. In particular, administration may be by direct injection to the cells. In another embodiment, administration may be intravenously, by hypospray or the use of PVP, an amorphous powder. Routes of administration include intramuscular, aerosol, oral, topical, systemic, olfactory, ocular, intraperitoneal and/or intratracheal.

Other features and advantages of the invention will be apparent from the following detailed description of the invention in conjunction with the accompanying drawings and from the claims.

Brief Description of the Drawings

Figure 1 represents the JTS-1 amino acid sequence and α-helix structure.

Figure 2 represents n-acyl tetrapeptides with membrane destabilizing activity.

Figure 3 represents α-helical peptides with lytic activity.

Figure 4 represents expression results of JTS-1 mediated expression in Skov-3, ML-3, Sol B, HCT-16α or CIT-26 cells.

Figure 5 represents expression results of JTS-1 mediated gene delivery.

Figure 6 is a representation of K_8 peptides and various R group substitutions.

Figure 7 is a representation of K_8 variations by changing side chain length and charged groups.

Figure 8 is a representation of pseudopeptides substituted at core lysine sequences of K_N to improve stability.

Figure 9 is a schematic for formation of pegylated K_N peptides.

Figure 10 is a representation of transfection efficiency using K_N peptides.

Figure 11 is a schematic formula of JTS/K_8 conjugates.

Figure 12 is a representation of transfection of C_2C_{12} myoblast cells with K_8/JTS-1/DNA complexes.

Figure 13 is a representation of transfection of 4Mbr-5 bronchus cells with K_6 or K_7/JTS-1/DNA complexes.

Figure 14 is a representation of target ligands used to direct delivery of JTS/K_8/DNA complex to the hepatocyte.

Figure 15 is a representation of target ligands containing carbohydrates for uptake by asialoglycoprotein receptor.

Figure 16 is a representation of target ligands for delivery of JTS/K_8/DNA to cells with mannose or mannose-6-phosphate receptors.

Figure 17 is a representation of RGD targeting ligands for delivery of JTS/K_8/DNA to connective tissue, wounds and for healing.

Figure 18 is a representation of ligands useful in delivery of JTS/K_8/DNA to hepatocytes.

The drawings are not necessarily to scale. Certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness. In addition, the drawings of PCT publication WO 93/18759 are hereby incorporated by reference.

Detailed Description of the Invention

The following are examples of the present invention using nucleic acid transporter systems with lysis and/or binding molecules for delivery of nucleic acid to a cell. These examples are offered by way of illustration and are not intended to limit the invention in any manner.

The following are specific examples of preferred embodiments of the present invention. These examples demonstrate how specific lysis agents release nucleic acid into the cellular interior. These examples also demonstrate how specific binding molecules stabilize and condense the nucleic acid for cell delivery. Furthermore, these examples demonstrate how surface and nuclear ligands can be used with a nucleic acid binding moiety to target nucleic acid into the cellular interior and/or the cell nucleus. Such targeted delivery is enhanced by use of the lysis agent and binding molecules. These examples include *in vivo* and *in vitro* techniques, various cellular or animal models and how nucleic acid can be inserted into cells. The utility of such nucleic acid transporter systems is noted herein and is amplified upon in the PCT publication WO 93/18759, by Woo et al., entitled "A DNA Transporter System and Method of Use," hereby incorporated by reference.

Below are provided examples of specific nucleic acid transporter systems that can be used to provide certain functionalities to the associated nucleic acid in the nucleic acid transporter system, and thus within a transformed cell or animal containing such associated nucleic acid. Those in the art will recognize that specific moieties of the nucleic acid transporter system can be identified as that containing the functional region providing the desirable properties of the nucleic acid transporter system. Such regions can be readily minimized using routine deletion, mutation, or modification techniques or their equivalent.

JTS Peptides, Analogs and Derivatives

In order to eliminate the use of adenovirus as an endosomal lysis agent, fusogenic or membrane disruptive peptides were designed which would increase the rate of delivery of nucleic acid from the endosome to the cell and ensure that higher concentrations of the endocytosed nucleic acid would be released and not degraded in the endosomes. A number of fusogenic/lytic peptides have been previously described, including the amino terminal sequence of the vesicular stomatitis virus glycoprotein and the synthetic amphipathic peptide GALA. Ojcius et al., *TIBS*, 16:225-229 (1991); Doms et al., *Membrane Fusion*, pp. 313-335 (Marcel Dekker, Inc., N.Y. 1991); Subbarao et al., *Biochemistry*, 26:2964-2972 (1987).

Short synthetic peptides from the hemagglutinin HA_2 subunit of influenza have been studied with artificial lipid membranes. Wharton et al., *J. Gen. Virol.*, 69:1847-1857 (1988). These peptides give both membrane fusion and leakage of liposomal contents similar to whole hemagglutinin molecules. However, the rates are quite slower.

In order to increase the low efficiency rate by endosomal lysis with influenza peptides, new peptides were created. In creating these new peptides for endosomal lysis, four factors were considered: (1) the content and spacing of the hydrophilic and hydrophobic amino acid residues along the α-helix to direct organized oligomer association of the peptides after their insertion into the membrane; (2) covalent attachment of the peptide to a binding molecule and preclusion of oligomer formation and the necessary aggregation; (3) sufficient aggregation of several oligomeric structures necessary to achieve lysis; and (4) presence of hydrophilic carboxyl and amino side chain and terminal groups to create the pH sensitive endosomal processing.

It is well known that the distribution of the amino acid side chains along the peptide chain determines the

secondary and tertiary structure of a protein. For membrane associating proteins, the amphipathic profile created by the hydrophobic and hydrophilic residues is a principal determinant of the function of the protein. Analysis of the region of the influenza hemagglutinin responsible for fusion of the viral envelope with the plasma membrane of cells reveals that a large hydrophobic surface is formed when the protein becomes α-helical (see discussion below).

In the present invention, a number of lytic peptides, *e.g.*, JTS peptides, have been designed and tested for endosomal lytic activity. In order for these peptides to be functional, they must have the following parameters. These peptides are amphipathic membrane associating peptides. These amphipathic peptides were designed as an α-helix, containing a sequence of amino acids such that the side chains are distributed so that the peptide has a hydrophobic and hydrophilic side. The hydrophobic side contains highly apolar amino acid side chains, while the hydrophilic side contains an extensive number of glutamic acids.

In general, the amphipathic membrane associating peptides usually contain 21 amino acids or fewer. The design criteria requires that the amino acids have a high probability of forming amphiphilic species. This can be exhibited in the secondary structure of the membrane associating peptides, *i.e.*, helices, turns, bends, loops, β-sheets, and their oligomeric aggregates and other super secondary structures defined in the literature, *e.g.*, helix-turn-helix. In addition, the amino acids should have a high probability of being found in an α-helix and a low probability of forming a β-sheet or turn structure. Leucine, lysine and glutamate are appropriate amino acids for such characteristics. For example, lysine positioned on the lateral face of the α-helix and glutamate residues opposite leucine provide optimal charge distribution for lipid interaction. Furthermore, lysines and glutamates

can be positioned to take advantage of potential helix stabilization. Helix dipole stabilization is optimized by removing the charge at the NH_2 and COOH-termini so NH_2 termini and COOH-terminal amides are useful. Such probabilities can be determined from secondary structural predictions or analogous methods to optimize secondary structural design. Unnatural amino acid which have been described for their propensity to induce helix structures in peptides are also used.

The hydrophobic or lipophilic face has a great effect on lipid-peptide interactions. Thus, the lipophilic face is modeled after peptides known to interact with lipids. Hydrophobic and lipid interactive residues (Ala, Leu, Met, Val, Phe, Trp, Tyr, Cys, Pro) when substituted on the lipophilic face either singularly or collectively promote a similar membrane associating effect. Similarly, an acid group and/or hydrophilic group (Glu, Gln, His, Lys, Gly, Ser, Asp, Asn, Pro, Arg) can be placed on the hydrophilic face to achieve the objective. The lipophilic and hydrophilic faces can also contain residues which promote lipid interaction and/or induce endosomal lysis at acidic pH. Such an interaction is not limited to an α-helix promoting residue since glycine and serine positioned on the hydrophilic face have been shown to favorably influence activity as seen with the examples below.

One in particular, the JTS-1 peptide, GLFEALLELLESL-WELLLEA, has a hydrophobic face which contains only strongly apolar amino acids, while the hydrophilic face is dominated by negatively charged glutamic acid residues at physiological pH values. At the amino terminus end, the JTS-1 peptide uses the Gly-Leu-Phe sequence at amino acid positions 1-2-3, respectively, as a fusogenic or membrane disruptive sequence. For increased pH sensitivity Glu is added at amino acid position 4. In addition, at positions 12-15, Ser-Leu-Trp-Glu is used as a lipid binding site. The remaining sequences are arranged to provide the hydrophobic and hydrophilic face of JTS-1. The helical

wheel of the amphipathic membrane associating peptide JTS-1 can be found in Figure 1. This figure shows the division of the hydrophobic and hydrophilic faces within the JTS-1 helical structure. Amino acids 16, 9, 2, 13, 6, 17, 10, 3, 14, 7 and 18 form the hydrophobic face. Amino acids 5, 12, 1, 8, 15, 4 and 11 form the hydrophilic face.

The following JTS peptides were constructed and characterized for lytic activity:

	Sequence	Molecular Weight	Parent Ions
JTS-1	GLFEALLELLESLWELLLEA	2301.8	2302.2
JTS-2	GLFEALLELLESLWELLLELYA	2578.2	2578.4
JTS-3	GLFEALLELLEELWELLLEA	2343.8	2342.9
JTS-4	GLFEALLELLEELWEALLEA	2301.8	2301.8
JTS-6	GLFEALLELLESLWELLLEAGGGGC	2633.2	2633.8
JTS-7	SLFEALLELLESLWELLLEA	2331.8	2332.4
JTS-8	GLFEALLELLESLYELLLEA	2278.8	2279.3
JTS-9	GLFEALAELLESLWEALLEA	2217.6	2218.3
JTS-10	GLFEALLELLESPWELLLEA	2285.8	2285.0
JTS-11	GLFEALLELLESLWEFLLEA	2335.8	2336.2
JTS-12	GLFEAILELLESLWELLLEA	2369.8	2302.2
JTS-12a	GLFEALLELWEA	1390.6	---
JTS-13	GLFEALLESLWEA	1477.7	1477.8
JTS-14	GLFEALLEILESLWELLLEA	2369.8	---
JTS-15	GLFEALLELWEA	1390.6	1390.8
JTS-16	GLFEALLELLESLWEA	1833.2	1834.0
JTS-17	GLFEALLELLESLWEFFLEA	2369.8	2370.4
JTS-18	GLFEALLELFESLWELLEA	2335.8	---
JTS-19	GLFESLLELLESLWELLLEA	2317.8	---
JTS-20	GLFEALLELLESLWELLKEA	2315.3	---
JTS-24	GLFEALLELLESLWELLLEAAEEA	2702.2	2703.4
JTS-10K8	GLFEALLELLESPWELLLEAGGGSG-SGSGSGSGYKAKKKKKKKKWK	4937.1	4937.7
JTS-15KPam	GLFEALLELWEAKNH$_2$$\epsilon$-Pam	1756.2	1757.0

JTS-16KBIO			
	GLFEALLELLESLWEAKNH$_2\epsilon$-BIOHX	2298.6	2300
JTS-1KBIO			
	GLFEALLELLESLWELLLEAKNH$_2\epsilon$-BIOHX	2642.8	---
acJTS-1	acGLFEALLELLESLWELLLEA	2345.8	2344.2
DMGJTS-1	Me$_2$GLFEALLELLESLWELLLEA	2350	---
desGJTS-1	LFEALLELLESLWELLLEA	2244.7	---
JTS-16KKCC14			
	GLFEALLELLESLWEAAAKLSKLEK-KLSKLEK	1833.2	---
GALA18	WEAALAEALAEALAEHLA	1879.1	1879.0
GALA30	WEAALAEALAEALAEHLAEALAEAL-EALAA	3030.6	3032.8

In addition to the above, n-acyl tetrapeptides with fusogenic or membrane destabilizing activity can be constructed. The structure of these is set forth in Figure 2. The tetrapeptide sequence when substituted with the appropriate amino acids as discussed above are capable of interacting with lipid bilayers and thereby destabilizing. The acyl chain can be lengthened or shortened depending on structure/function requirements.

Furthermore, shorter α-helical peptides were also synthesized with the above design motifs in mind to retain the lytic properties as discussed above. Figure 3 shows the helical wheels of smaller fusogenic peptides. For example, LLEKLLEWLE (number IV in Figure 3) is a shorter α-helical peptide with lytic properties. Leucine is used for hydrophobic properties and α-helical movement. Glutamic acid residues are used for lytic activity. These residues also have the propensity to form an α-helical structure at pH 4.0. Furthermore, a COOH-terminal amide is used to provide helix-dipole optimization. When in an α-helical structure the hydrophobic face appears at positions 4, 7, 3, and 10.

To provide the Gly-Leu-Phe fusogenic or membrane disruption activity to the above α-helical peptide in Figure 3, the peptide was lengthened to an 11-mer. Adding the additional amino acid to form the following peptide, Suc-GLFKLLEEWLE, allowed the activity of the three glutamic acids to be retained. In addition, the peptide was succinylated at the amino terminus to afford an i to i+4 salt bridge with lysine which is designed to stabilize the helix.

Synthesis, Purification and Characterization of JTS-1 Peptides

JTS-1 peptides are synthesized by the solid phase method as developed by Merrifield et al., *Solid Phase Peptide Synthesis*, Academic Press (N.Y. 1980). In addition, a modified polystyrene (Sparrow, *J. Org. Chem.*, 41:1350-1353 (1976)) and/or polyamide resin (Sparrow et al., *Int. J. Peptide Prot.*, 38:385-391 (1991)) with fast HBTU/HOBT coupling is used. It should be noted that the following procedure was used to synthesize INF-7 as well. The procedures involved in solid phase synthesis include: (1) attachment of the protected carboxyl terminal amino acid to the solid support through the oxymethyl-phenylacetamide linkage; (2) deprotection of the N-terminal amino group; (3) neutralization of the amino group; (4) coupling of the next N-protected amino acid to the peptide resin; and (5) after completion of the synthesis, removal of the peptide from the solid support.

Specifically, the carboxyl terminal amino acid protected with the N-^{t}butyloxycarbonyl group is esterified to bromomethyl-phenylacetic acid and coupled directly to aminomethyl-polystyrene resin or to a resin containing a long spacer chain between the point of attachment and the polystyrene backbone or directly to the amino-propyl polyamide resin. In the fast HBTU/HOBT synthesis protocol the above procedure is modified as follows to give a total program time of 45 minutes. Trifluoroacetic acid (100%)

is used to deprotect the amino group in 6 minutes. The resulting salt is neutralized by the excess diisopropylethylamine used to activate the N-^{t}butyloxycarbonyl amino acid with HBTU/HOBT in dimethylformamide ("DMF"). This combines the coupling and neutralization steps. The coupling reaction is allowed to proceed for 15 minutes and the resin washed extensively with DMF and dichlormethane ("DCM"). These steps are then repeated until the sequence of interest has been synthesized.

To protect side chains, the following are used: (1) 2,6-dichlorobenzyl for the hydroxyl of tyrosine; (2) benzyl for the hydroxyl of serine and threonine; (3) benzyl esters for the β- and y-carboxyl of aspartic and glutamic acids; (4) 2-chlorobenzyloxycarbonyl for the ϵ-amino of lysine; (5) p-methoxybenzyl or acetamidomethyl for the sulfhydryl of cysteine; (6) formyl for the indole of tryptophan; (7) benzyloxymethyl for the imidazole of histidine; (8) trimethyl benzenesulfonyl for the guanidino of arginine; and (9) xanthanyl for the amido of glutamine and asparagine.

The peptide is cleaved from the solid support by treatment of 1 g of resin with 60 ml of anhydrous hydrogen fluoride containing 10% anisole and 1% ethanedithiol for 30 minutes at 0°C. In the case of peptides containing arginine, the cleavage is performed at -20°C for 3 hours. The hydrogen fluoride is evaporated under vacuum at 0°C and the peptide precipitated with ether. The peptide and resin are filtered off and washed with ether. The peptide is then extracted with trifluoroacetic ("TFA") (3 x 30 ml) and the TFA evaporated under vacuum. The peptide is precipitated with ether and the precipitate collected by centrifugation. The precipitate is suspended in 10 ml 1 M TRIS and 6 M GnHCl. An additional 30 ml of 6 M GnHCl is added to completely dissolve the peptide. The pH of the solution is adjusted to 8.0. The peptide is desalted on a column of BioGel P-2 equilibrated in 0.1 M ammonium

bicarbonate for the lytic peptides. The peptide fractions are located by absorbance at 254 nm and 280 nm, pooled and lyophilized. The lyophilized peptide is dissolved in 25 ml of 6 M GnHCl for the lytic peptides.

A_{280} is used to determine peptide concentration. This is performed in 6 M guanidine HCl for JTS-1 or other peptides so that aggregation is not observed. The following molar extinction coefficient is used for JTS-1 in 6 M guanidine HCl - 5600.

The peptide is purified by reversed phase HPLC. The following procedures were used. The peptide (50-100 mg in 5 ml of 6 M GnHCl) is diluted with 20 ml of 0.1 M ammonium phosphate in 6 M GnHCl, pH 6.7 and the pH confirmed. This solution is pumped onto a 2.5 x 25 cm Vydac C4 column (214TP152022; 300 Å pore size) equilibrated in 0.01 M ammonium phosphate at a flow rate of 20 ml/min. Two buffers are used to elute the peptide, Buffer A (0.01 M ammonium phosphate in ddH_2O, pH 6.7) and Buffer B (2-propanol 100%). The peptide is eluted with a linear gradient between Buffer A and 30% Buffer B, and Buffer A and 50% Buffer B. The gradient program used is Buffer A to 50% Buffer B for 45 minutes, with retention time being 40-41 minutes. The peptide is detected by absorbance at 254 and 280 nm.

The peptide containing fractions are pooled, the pH adjusted to 8.0 with ammonium hydroxide and desalted on a BioGel P-2 column equilibrated in 0.1 M ammonium bicarbonate. The peptide containing fractions are pooled and lyophilized. The peptide is dissolved in water after lyophilization. Diluted ammonium hydroxide is added to adjust the pH to 7.5 in order to completely dissolve the peptide.

The following criteria were applied to evaluate the purity of the synthetic products: 1) analytical high performance liquid chromatography using a linear gradient between 0.01 M ammonium phosphate, pH 3.0 or pH 6.8 and 2-propanol and 0.1% TFA and 2-propanol, 2) fast atom

bombardment or electrospray mass spectrometry, 3) amino acid analysis for correct composition, 4) automatic amino acid sequencing, and 5) capillary electrophoresis. For amino acid analysis of the peptides for correct composition, decomposition and purity, quantitation of the molar ratios of the peptide components determines peptide purity. With fast atom bombardment mass spectrometry ("FAB") purity, molecular weight can be determined. A single peak for JTS-1 occurs at the expected mass of 2302 amu.

Secondary structure is determined by circular dichroic and FTIR spectroscopy. These standard methods are used to confirm the secondary structure of JTS-1.

Since guanidinium HCl is used to solubilize the JTS-1 during purification, guanidinium contamination must be tested. Guanidinium contamination in JTS-1 is detected by the Sakaguchi method for determining arginine (guanidinium). This method is well known in the art.

Synthetic Membrane Disruptive Behavior of JTS-1

In order to test the synthetic membrane disruptive behavior of JTS-1, a comparison of turbidity was performed with liposomes containing either JTS-1, polyvinylpyrrolidone ("PVP") or Tween80 (a detergent). The liposomes were made from dimyristoylphosphatidylcholine ("DMPC") by methods well known in the art. Phosphatidylcholine vesicles containing calcein were prepared by sonication. Briefly, 10 mg/ml phosphatidylcholine was dried down under a stream of nitrogen. The lipid was resuspended in 100 mM calcein (adjusted to pH 7.3 with sodium hydroxide) and sonicated with a probe sonicator for 20 minutes in an ice bath. Liposomes were separated from unentrapped calcein using a Sephadex G25 column. Calcein release was measured at 520 nm (excitation at 470 nm) using a fluorescence spectrophotometer. For leakage assays, liposomes were diluted 1000-fold in 150 mM NaCl, 15 mM sodium citrate, pH

7.0 or 5.0. Peptide was added at a concentration of 1 μg/ml. Fluorescence was measured before and 10 minutes after addition of peptide. 100% leakage was determined by adding Triton X-100 to a final concentration of 0.5%. The turbidity was monitored using apparent absorbance measurements at 400 nm on a visible-wavelength spectrophotometer. This experiment was performed at various pHs.

Initially, all liposomes were turbid. The clearance, *i.e.*, decrease in turbidity, as a function of time after adding the test samples to the liposomes was then measured. Both JTS-1 and Tween80 showed fusogenic or membrane disruptive behavior, *i.e.*, decrease in turbidity, whereas PVP did not. JTS-1 lysed phosphatidylcholine liposomes to a greater extent at pH 5.0 than at pH 7.0.

It should be noted that Tween80 was used as a positive control. Tween80 is a surface active agent which concentrates at oil-water interfaces causing an emulsifying action. Such properties allow Tween80 to disrupt membranes of synthetic liposomes. PVP was used to see if there was any activity. PVP is an amorphous powder, is compatible with hydrophobic and hydrophilic residues. Although it can be used as a detergent, PVP has colloid protective properties. These properties allow PVP to act as a surface-active substance that prevents the dispersion of a suspension, *i.e.*, liposomes, from coalescing by forming a thin layer on the surface of each particle. In addition, the above assay was also performed using INF-7 instead of JTS-1 (see description of INF-7, below). INF-7 showed pH dependent membrane activity on phosphatidylcholine liposomes. The activity of INF-7 was approximate 8-fold lower than that of JTS-1.

Hemolysis Activity of JTS Peptides

The JTS peptides were studied for their ability to lyse erythrocytes. Erythrocytes were isolated by methods known in the art. Hemolysis assays were performed

according to the literature. Erythrocyte lysis assays have been previously used to determine the membrane activity of bacterial toxins and membrane active peptides. Human erythrocytes were washed three times with phosphate buffered saline and were resuspended in 150 mM NaCl, 15 mM sodium citrate, pH 7.0 or 5.0, at a concentration of $7x10^7$/ml. Peptides were diluted serially in 150 mM NaCl, 15 mM citrate pH, 7.0 or 5.0, in a 96-well plate. Next, 75 μl erythrocyte suspension was added to each well. The plates were incubated at pH 7.0 and pH 5.0 for 60 minutes at 37°C with occasional shaking. Unlysed erythrocytes were pelleted and the extent of hemolysis was determined visually. One hemolytic unit (HU) was defined as the amount of protein necessary to induce >50% hemolysis. All hemolysis assays were performed in duplicates.

JTS-1 was hemolyticly active at pH 5.0 and not at pH 7.0. At pH 5.0, the specific hemolytic activity was $1x10^7$ erythrocytes lysed per μg of peptide. JTS-3 had a specific hemolytic activity at pH 5.0 of $8x10^7$ erythrocytes lysed per μg of peptide, whereas JTS-9 was $3x10^7$ erythrocytes lysed per μg of peptide.

In a separate hemolysis assay, it was determined that the hemolysis activity of JTS peptides decreased as the pH increased. Hemolysis activity was stronger at pH 5.0 and is undetectable at pH 7.0. These studies show that the JTS-peptides exert enhanced lysis activity at pH 5.0.

Hemolysis Assay Comparing JTS-1 and Influenza ("INF-7") Peptide

JTS-1 and INF-7 peptides were studied for their ability to lyse erythrocytes. INF-7 is the active region of HA_2, influenza hemagglutinin, responsible for fusion of the influenza viral envelope with the plasma membrane of cells. INF-7 has the following amino acid sequence, GLFEAIEGFIENGWEGMID. Amino acid numbers 5, 16, 9, 2, 13, 6, 17, 10, 3 and 14 of the helical wheel form the

hydrophobic face. Amino acid numbers 7, 18, 11, 4, 15, 8, 1 and 12 of the helical wheel form the hydrophilic face.

Erythrocytes were isolated by methods well known in the art. Serial dilutions of INF-7 and JTS-1 peptides were incubated with washed human erythrocytes at pH 7.0 and pH 5.0. After one hour, the unlysed erythrocytes were pelleted. The concentration at which 50% lysis occurred was determined by visual reading. INF peptides showed no hemolytic activity at either pH 7.0 or pH 5.0. Just as above, JTS-1 was hemolyticly active at pH 5.0 and not at pH 7.0. At pH 5.0, the specific hemolytic activity was $1x10^7$ erythrocytes lysed per μg peptide.

JTS Peptide-Liposome Leakage Assay

Liposome membrane activity was measured by testing liposomal leakage. This assay measures the release of calcein, a fluorescent dye, from phosphatidylcholine ("PC") vesicles. Briefly, calcein is encapsulated into liposomes by well known procedures at a concentration where the fluorescence of the dye is greatly reduced (self-quenching). When the liposomes are destroyed by the lysis agent, the fluorescent dye leaks out of the liposomes and is diluted in the incubation buffer. This causes a great increase of fluorescence (dequenching) which can be followed in a fluorescence spectrophotometer.

Liposomes were incubated with monomeric forms of JTS-1, JTS-3 and JTS-9 peptides at a concentration of 0.5 μg/ml in a sodium-citrate buffer with a pH ranging from 5.0 to 7.0. Before and 5 minutes after the addition of the lysis agent peptides, the fluorescence was determined. The fluorescence corresponding to 100% leakage was determined by complete lysis of the liposomes with a detergent (Triton X-100; final concentration 0.5%) and the values obtained were plotted as percentage leakage.

JTS-1 lysed phosphatidylcholine vesicles, as well as erythrocytes, in a pH-dependent manner. No membrane

activity was observed at pH 7.0. A sharp increase of membrane activity was observed at a pH lower than 6.0.

In comparison, use of the influenza fusion peptide, INF-7, showed pH-dependent membrane activity on liposomes. In contrast to JTS-1, only very little hemolytic activity was observed. Furthermore, the JTS-9 peptide was shown to be more potent than the activity of JTS-1 and JTS-3.

JTS-1 Mediated Expression in Cell Lines

The ability of JTS-1 to mediate expression in cells *in vitro* was tested using DNA complexes containing a CMV-β-galactosidase expression vector with transferrin/poly-L-lysine and unmodified poly-L-lysine to create a positive particle. The cell lines tested were obtained from ATCC and cultured by well known methods in the art.

Poly-L-lysine ("PLL") Mwt. 20,500, was coupled to transferrin in a 1 to 2.0 ratio by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide ("EDC") at pH 7.3. The reaction was incubated for 24 hours at room temperature after which it was concentrated and resuspended in 2 M Guanidine-HCl, 50 mM HEPES, pH 7.3, and fractionated by gel filtration on a Superose 6 column with a Fast Protein Liquid Chromatography system ("FPLC"). Once the conjugate was made and purified, fractions from the FPLC were analyzed on an SDS-PAGE gel to determine those fractions that contained modified transferrin ("TF/PLL conjugate") only. These fractions were then pooled and dialyzed against 150 mM NaCl, 20 mM HEPES, pH 7.3 prior to complex formation.

The DNA plasmid CMV/β-gal containing the *E. coli* β-galactosidase gene under the control of the CMV enhancer and promoter was used as a reporter gene. The plasmid was isolated and purified by double CsCl banding. This plasmid has been thoroughly described in the art. In addition, unmodified poly-L-lysine was added to help create a positive particle. The JTS-1 peptides were added and bound to the DNA complex through ionic interactions.

Conjugate/DNA complexes were prepared by diluting the conjugate in 150 μl of HBS (150 mM NaCl per 20 mM HEPES, pH 7.3) and diluting 6 μl of DNA, in 350 μl of HBS. The diluted DNA was added directly to the diluted conjugate while mixing. The reaction was allowed to incubate at room temperature for 30 minutes before analysis. Immediately following the incubation, all complexes were analyzed on 0.8% agarose gels and electrophoresed in TBE.

Six μg of DNA complex was incubated with $3x10^5$ tissue culture cells. Before adding the complex to the tissue culture cells, the complete media was removed and replaced with 1 ml of Low Glucose DMEM containing, 5 mM Ca^{2+} and 2% fetal calf serum. After a 2 hour incubation at 37°C, 1.5 ml of complete media was added to the tissue culture cells and the incubation continued for 24 hours at 37°C.

After 24 hours, analysis of β-galactosidase (β-gal) activity was performed by staining the cells, using X-gal as a substrate. The following procedures were used to analyze β-galactosidase activity. The cells are washed with 1X PBS twice. The cells are then fixed for 15 minutes in a solution containing 1% glutaraldehyde (from 50% stock), 100 mM sodium phosphate buffer, pH 7.0, and 1 mM $MgCl_2$.

The cells are washed with 1X PBS twice and incubated at 37°C for 30-60 minutes with the solution containing 0.2% X-gal, 10 mM sodium phosphate buffer, pH 7.0, 150 NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6 3H_2O$ and 3.3 mM $K_3Fe(CN)_6$.

The staining solution is removed and the cells washed with 1X PBS twice. The stained cells can be identified under a phase-contrast microscope (400x). To quantify the actual amount of β-galactosidase produced, ONPG can be used as a substrate with aliquots of cell extracts.

Figures 4 and 5 show the expression results. With MCA-26 cells, up to 40% of the cells were stained blue; 3T3 cells, 30%; Sol 8 cells, 20%; 4MBR-5, 50%; 293 cells,

90%; human fibroblast, 30%; and SKOV3 cells, 1%. Without peptides no blue cells were observed.

In addition to the above, the expression of JTS-1 mediated expression was compared with an INF-7 peptide using the same procedures as above and transfecting Sol 8 myoblast cells. Up to 5% of the cells were positively stained blue where JTS-1 was part of the complex. In the absence of peptides, no cells were stained blue. With INF-7 peptides as part of the complex, only a few blue cells (<0.01%) were observed.

Gene Expression Using Various Ratios of JTS-1 to Transferrin/PLL

DNA complexes were made by condensing a CMV-β-galactosidase expression vector with transferrin/poly-L-lysine and unmodified poly-L-lysine to create a positive particle. The same procedures as described above were used. The lytic peptides were then added and were bound to the DNA complex through ionic interactions as discussed above. Various concentrations of DNA complex (5-20 μgm) and ratios of JTS-1 to transferrin-PLL (2.5-42.5 μgm) were incubated with $3x10^5$ Sol 8 myoblast cells. In addition, in one series of experiments 12-18 μgm of PLL was also added. After 24 hours, β-galactosidase activity was determined. Significant β-galactosidase activity was observed at all ratios of JTS-1/transferrin-poly-L-lysine tested. In almost all cases, increasing JTS-1 concentration enhanced transfection and therefore expression of β-galactosidase.

Human Fibroblast Uptake and Degradation of LDL/JTS Complexes

To form the ^{125}I-LDL/JTS complexes, ^{125}I-LDL was dissolved in dimethylsulfoxide and incubated with 10-fold excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide ("EDC") for 30 minutes at room temperature. A 30-fold excess was added to JTS peptides (89 KBg/μg) in phosphate buffer and incubated for 4 hours at room temperature. The

reaction was quenched with a 50-fold excess of ethanolamine. Free JTS was separated from the ^{125}I-LDL/JTS complex by passing the reaction mixture over a Sephadex G-25 column equilibrated with phosphate buffered saline, at pH 7.4.

A 10:1 molar ratio of JTS peptides bound to ^{125}I-LDL was incubated with human fibroblasts for 5 hours at 37°C. Fibroblasts were isolated and cultured by using methods well known in the art. JTS-1, JTS-3, JTS-8 and JTS-9 peptides were used for the uptake/degradation studies. As a control, ^{125}I-LDL without JTS peptides were also incubated with the same fibroblast and analyzed accordingly.

After incubation for 5 hours, cells were harvested to determine internalized radioactivity. Cells were washed once with ice cold PBS, followed by a 30 second wash with ice cold acid saline (0.15 M NaCl, adjusted to pH 3.0 with glacial acetic acid) to remove surface bound LDL and then harvested by scraping in ice cold PBS. The cells were pelleted, washed with PBS, and then dissolved in 0.1 M NaOH. One-half was used for protein determination (BCA-Protein assay) and the other half was counted in a scintillation counter. For competition, a 100-fold molar excess of LDL was added prior to incubation with the ^{125}I-LDL-JTS complexes.

Binding and uptake of ^{125}I-LDL was not affected, while JTS peptides decreased degradation by 50%. To insure LDL uptake was specific for the JTS peptides, the experiment was repeated in the presence of 20-fold excess cold LDL. Under these conditions, more than 90% of the cellular uptake of ^{125}I-LDL-JTS was inhibited. Cells receiving ^{125}I-LDL showed intense perinuclear fluorescence. Most cells receiving both JTS peptides and ^{125}I-LDL had diffuse intracellular fluorescence. Using chloroquine to block endosome acidification, fluorescence was perinuclear, the same as that observed without JTS peptide.

The same studies were performed with HAEC cells. HAEC were isolated and cultured using methods well known

in the art. Binding and uptake of ^{125}I-LDL was not affected, while JTS-1 and JTS-8 peptides decreased degradation by 30% depending on the amount of JTS peptide used. To show that LDL uptake was specific for the JTS peptides, the experiment was repeated in the presence of 20-fold excess cold LDL. Under these conditions, more than 90% of the cellular uptake of ^{125}I-LDL-JTS was inhibited.

K_N Peptide Analogs and Derivatives

A variety of peptides with the formula $YKA(K)_NWK$, wherein N = 1-40, have been synthesized. This general structure for K_N peptides, more specifically K_8, is shown in Figure 6. The lysines (*i.e.*, "K") act as the binding molecule. The tyrosine partially contributes to the A_{280} and also allows for the iodination for tracking *in vitro*. Tryptophan increases the stability of interaction with DNA through intercalation and also provides a fluorophore which quenches upon interaction with the DNA. When R in Figure 6 is tryptophan, smaller particles are obtained and improved transfection occurs. In addition, the R group can also be substituted with other R groups to achieve the same effects. See Figure 6 for additional R groups for improving peptide activity. Alanine provides a linker that allows the tyrosine and nearest neighbor lysine residues to be wrapped around the DNA in a more helical fashion resulting in a more stable complex.

Extensive characterization of the interactions of these peptides with DNA were performed for each of the peptides. In these experiments, the dependence of time, peptide concentration, and number of lysines in the peptide on the size of the peptide-DNA complexes formed were examined. Condensates formed between K_N peptides and DNA polymers have a higher propensity to aggregate when the relative charge ratios of peptide lys:DNA phosphates are near 1:1. This is more pronounced for the lower molecular weight peptides. Higher molecular weight

peptides tend to result in particles that are smaller and more monodisperse. At a constant lys:phosphate ratio, condensed particle size tends to increase with increasing concentrations of DNA.

Variations to optimize K_8 activity are also useful. The K_8 peptide contains an octamer of polylysine. To optimize the nucleic acid condensing activity of the cationic peptide, variations, as shown in Figure 7, of the side chain length and charged groups are made. Modifications introduced at the core cationic oligomer decrease or increase the number of methylenes placed between the side chain cationic group and the peptide backbone. For instance, Figure 7 shows the use of a peptide backbone spacer that varies the distance between cationic groups, where the cationic group is the α-amino group of the substituted amino acid, *e.g.*, for K_8 it would be the α-amino of lysine. Likewise, NH_2- and COOH-terminal substitutions and deletions can also be used to optimize DNA binding of the molecule. For example, variation of a NH_2 or COOH terminal acyl group may yield enhanced activity. Functionalities of the charged or cationic groups include guanidinium, amine or imidazole. Furthermore, variations of the NH_2- and COOH- termini include esters, acyl groups and amides, as well as deletions.

In addition to the above, amino charged groups can be substituted in the peptide backbone for condensing the associated nucleic acid. Pseudopeptides of the formula $\psi[CH_2NH]$ when substituted within the core lysine sequences (see Figure 8), improves stability and enhances electrostatic interactions with the nucleic acid phosphates. Other possible substitutions using $\psi[(CH_2)_nX]$ where X is a heteroatom can help optimize the intermolecular ionic interactions important for condensing the nucleic acid.

Other modifications include pegylated K_N peptide analogs to increase plasma half-life, resistance to

degradation, solubility and decreased antigenicity and immunogenicity. Figure 9 outlines a scheme for pegylation of lysine. Once the Fmoc-Lys-N-(ε-PEG)-OH is synthesized, it can be used for the solid phase synthesis of K_N-pegylated peptides.

The following K_N peptides were constructed and characterized:

Condensing Peptides		Molecular Weight	Parent Ions
K4	YKAKKKKWK	1207.5	1207.8
K5	YKAKKKKKWK	1335.7	1336.1
K5FK	YKAKKKKKFK	1296.7	1296.5
K5LK	YKAKKKKKLK	1262.6	---
K6	YKAKKKKKKWK	1463.9	---
K7	YKAKKKKKKKWK	1592.1	---
K8	YKAKKKKKKKKWK	1720.2	1719.7
FK8	YKAKKKKKKKKFK	1681.2	1680.1
R4K8	YKAKKKRKKKKWK	1748.3	1747.4
R8	YKARRRRRRRRWR	1972.3	---
GSK8	GSGSGSGSGSGYKAKKKKKKKKWK	2498.1	---
CGSK8	CGSGSGSGSGSGYKAKKKKKKKKWK	2601.2	---
WK8	WKAKKKKKKKKWK	1743.3	1742.8
WK10	KWKKKKKKKKKKWK	1928.6	1928.4
K10	YKAKKKKKKKKKKWK	1977	---
K12	YKAKKKKKKKKKKKKWK	2233	---
KA20	YKAKAKAKAKAKAKAKAKAKAKAKA-KAKAKAKAKAKAKAKAKAKWK		
K40	YKAKKKKKKKKKKKKKKKKKKKKKKK-KKKKKKKKKKKKKKKKKKWK	5822	5820.6
KKCC14	KLSKLEKKWSKLEK	1744.1	1744.3
KKCC21	KLSKLEKKLSKLEKKWSKLEK	2570.6	2571.2
A4K10	KAKKAKKKAKKAKWK	1770.3	1769.8
S4K10	KSKKSKKKSKKSKWK	1834.3	---
SSK8 ("Short NLS")	YKAKKKKNH$(CH_2)_2SS)CH_2)_2$COK-KKKWK	1720.2	---

SHNLSK6	STPPKKKRKVEDPKDFPSELLSAKKK-KKKWK	4044.2	---
SHNLSK8	STPPKKKRKVEDPKDFPSELLSAYKA-KKKKKKKKWK	4300.2	4299.3

("Long NLS")

LGLSK6	SSDDEATADSQHSTPPKKKRKVEDPK-DFPSELLSKKKKKKWK	4925.7	4924.9
LGLSK8	SSDDEATADSQHSTPPKKKRKVEDPK-DFPSELLSAYKAKKKKKKKKWK	5544.5	---

K40 - Short NLS (attached at the α-amino of Tyr and ε-amino group of Lys-2)

K40 - Long NLS (attached at the α-amino of Tyr and ε-amino group of Lys-2)

Synthesis, Purification and Characterization of K_N Peptides

Peptides were synthesized as discussed above for the JTS peptides. In addition, the peptides were also cleaved from the solid support as discussed above for JTS peptides with the following changes. After precipitation of the peptide with ether, the precipitate is suspended in the same solution; however, water replaces the 6 M GnHCl and the pH is adjusted to 3.5 instead of 8.0 as used above. In addition, the peptides are desalted on the BioGel P-2 column equilibrated with 5% acetic acid instead of 0.1 M ammonium described above for the JTS peptides. After the collected peptide is lyophilized, it is dissolved in 25 ml of water instead of 6 M GnHCl for the lytic peptides.

A_{280} is used to determine peptide concentration. This is performed in water for K_N peptides so that aggregation is not observed. The following molar extinction coefficient is used for K_8 - 6860.

The peptide is purified by reversed phase HPLC. The peptide (50-100 mg in 5 ml of water) is diluted with 20 ml of 1% TFA and the solution pumped onto a 2.5 x 25 cm Vydac C18 column (218TP152022, 300 Å pore size) equilibrated in 0.1% TFA (Buffer A). The peptide is eluted with a linear gradient between 0.1% TFA and 0.1% TFA, 10% 2-propanol

(Buffer B) at a flow rate of 20 ml/min. The gradient used is 100% A to 10% B for 30 minutes.

Then the column is washed with 10% B to 90% B in 5 minutes, 90% B for 5 minutes, then 90% B to 100% A in 5 minutes. The retention time is 6.0 to 6.8. The peptide is detected by absorbance at 254 and 280 nm. The peptide containing fractions are pooled, frozen and lyophilized. The peptide is then dissolved in water. Purity is confirmed by analytical reversed phase HPLC.

The purity and molecular weight is also determined by electrospray mass spectrometry ("ESMS"). ESMS has been performed on K_8 peptides. A single peak for K_8 occurs at the expected mass of 1720 amu. Decomposition and purity is also determined by amino acid analysis of the peptides. Quantitation of the molar ratios of the peptide components determines peptide purity.

Secondary structure is determined by circular dichroic and FTIR spectroscopy. These standard methods are used to confirm the secondary structure of K_8 and other K_N peptides.

K_N Cytotoxicity Studies

Prior studies using poly-L-lysines with DNA/protein complexes have been shown to be toxic in nM concentrations to living cells. This limits the general applicability of such poly-L-lysines. Such peptides were 50-200 in average chain length. In order to avoid such toxicity, K_N peptides were constructed. These shorter polylysine peptides were synthesized as discussed above, such as K_8 which contains a central cluster of eight lysines. To show that K_N peptides are not cytotoxic to cells, HepG2 cells were incubated with K_8 and with PLL. HepG2, a hepatocyte cell line, were cultured using standard methods known in the art. HepG2 cells were incubated at 37°C for 24 hours with increasing concentrations of K_8 or poly-L-lysine (100 mer), after which viable cells were counted. Poly-L-lysine concentrations of greater than 0.1 μM led to complete cell

death of HepG2 cells. In contrast, no cytotoxicity was observed for up to 100 μM of K_8, the highest concentration tested. This indicates that K_8 is at least 1000-fold less toxic than poly-L-lysine for HepG2 cells.

DNA/K_N Transfection Efficiency in C_2C_{12} Myotubes

In order to determine K_N peptide transfection efficiency, C_2C_{12} myotubes were transfected with K_N/DNA complexes.

DNA was added to each well in a volume containing 300 μl per well in Fisherbrand culture tubes. The K_N peptide is added to each well with DNA and then vortexed before the two solutions mix. Samples then set for at least 30 minutes.

If JTS-1 peptide is to be added as in the studies below, then JTS-1 is added to each of the DNA-K_N peptide samples and vortexed before the two solutions mix. The samples then set for at least 30 minutes.

C_2C_{12} myotubes in DMEM containing 10% FBS are incubated for 30-50 minutes prior to transfection. Then, 300 μl of the solution complex is added to each well in a 24-well plate and incubated for 5 hours at 37°C. After 5 hours, 1 ml of DMEM containing 10% FBS is added.

Cell extracts are prepared by adding 100 μl lysis solution to each well (24 well-plate). Cells are transferred to a microfuge tube and centrifuged for 10 minutes at 4°C to pellet any debris. Supernatant is transferred to a fresh microfuge tube and cell extracts are frozen at -70°C for future use.

The cell extracts are diluted 50 times by ddH_2O. Cell extracts in 5-20 μl aliquots are diluted so that the total volume is 20 μl. Reaction Buffer (200 ml) is added with sample to luminometer tube and mixed, and then incubated at room temperature for 2-3 hours. Then, 300 μl of Accelerator is injected and the sample counted.

The cells are harvested two days later and viewed for β-Galactosidase activity. Chemiluminescent assays are then performed as discussed.

Figure 10 is a graph showing the effects of K_N peptide molecular weight on transfection efficiency in C_2C_{12} myotubes. Of the peptides used (K_5, K_6, K_7 and K_8), K_8 provided the highest transfection efficiency in C_2C_{12} myotubes.

Nuclear Localization Sequence ("NLS")/K_N

In addition to the above characteristics, K_8 also has nuclear targeting capabilities. The nuclear localization ligand containing peptide GYGPPKKKRKVEAPYKA$(K)_N$WK was used to form a nuclear binding molecule by the same procedures as described above. The tyrosine can be used for incorporation of ^{125}I to quantify binding parameters and to determine stoichiometry of the DNA complex. Binding of the peptide to DNA quenches tryptophan fluorescence and allows the kinetics and thermodynamics of complex formation to be determined. The function of the EAP sequence is to extend the nuclear localization sequence, GYGPPKKKRKV, at right angles to the lysine backbone. The peptide is homogenous by reversed phase HPLC and has the expected molecular weight, determined by electrospray mass spectroscopy.

Coupling or Association of JTS-1 to K_8

JTS and K_8 can be associated by covalently linking JTS-1 and K_8 together to form a bifunctional condensing/endosomal peptide as depicted in Figure 11. JTS-1 peptides were combined with K_8 peptides at a concentration of 16 μM, along with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) at a final concentration of either 130 μM (low EDC) or 2600 μM (high EDC) in a final volume of 4 ml. After incubation on ice for four hours after which time the unreacted components were removed by ultra-centrifugation (150,000xg) for 18

hours on a CsCl gradient at a CsCl concentration of 1.35 g/ml. JTS-1/K_8 complexes can be shown in Figure 11. The DNA used in complex formation was the plasmid pCMV/βGal, which contains the β-galactosidase gene under the control of the CMV promoter. This complex was constructed as follows.

The JTS-1/K_8/DNA complexes were made by adding 10 μg of DNA, in 250 μl of HBS, to the JTS-1/K_8 conjugate in 250 μl, with continuous mixing, followed by incubation at room temperature for 30 minutes. The JTS-1/K_8 conjugate was synthesized and purified as described above. Sufficient JTS-1/K_8 conjugate to neutralize 75% of the charge on the DNA molecule was used. After complex formation, the complexes were either analyzed by electron microscopy or used with the cell line for analysis.

In addition to the above, LDL-receptor gene/K_8/JTS-1 complexes were also constructed as described above. LDL/K_8/JTS-1 complexes were transfected with fibroblasts using the transfection procedures above. These complexes led to functional expression of LDL-receptor in Watanabe fibroblasts (see below). DNA/K_8/JTS-1 complexes mediated high levels of gene expression in a variety of cell lines (see below).

JTS-1 and K_8 can also be associated noncovalently. Nucleic acid, K_8 and JTS-1 were associated by calculating different ratios of DNA, K_8 and JTS-1 by only considering the negative charges of DNA (phosphate groups) and JTS-1 (5α-carboxylic groups), and the positive charges of K_8 (10ϵ-amino groups). Six μg of DNA in 500 ml 250 mM sucrose were mixed at a phosphate to amino group ratio of 1:2, 1:3 or 1:4. After incubation for 30 minutes at room temperature, 7-38 mM JTS-1 was added to create either positively charged, neutral or negatively charged DNA/K_8/JTS-1 complexes. Positively charged complexes had an overall +/- charge ratio of 0.66 to 0.7, neutral complexes a ratio of 1:1 and negatively charged complexes a ratio of 1:1.5. DNA/K_8/INF-7 could also be prepared as

above. For effective transfection, the preferred embodiment utilizes a relative charge ratio of nucleic acid/K_8/JTS-1 at 1/x/y, where $x > 1$, and $0.25 < y < 2$ ($x = K_8$; y = JTS-1). Optimal transfections occur when using $2 < x < 6$ and $0.75 < y < 2$.

The Effect of pH on Particle Size of the DNA/K_8/JTS-1 Complex

The following is useful in characterizing the DNA/K_8/JTS-1 complex. The effects of pH on the particle size of 100 μg/ml pDNA/K_8/JTS-1 complex was examined. The complex was formed as discussed above. When the pH was dropped to below pH 7, JTS-1 caused the complex to precipitate. When the pH was above 9, K_8 deprotonates and loses its ability to bind DNA efficiently. Thus, the complex falls apart. Therefore, the optimal pH for the complex is between pH 7-9.

The Effects of NaCl Concentration on the Particle Size of DNA/K_8/JTS-1

The following is useful in characterizing the DNA/K_8/JTS-1 complex. From 0.1-0.4 M NaCl, increasing salt concentration reduces electrostatic repulsion between complexed particles by lowering the Debye length through ion atmosphere screening. This increases the chances of particle coagulation. The particle is completely decomplexed by 0.4 M NaCl as indicated by a large decrease in counts per second.

The Effects of Different Isotonic Solution on the Particle Size of the DNA/K_8/JTS-1 Complex

The following is useful in characterizing the DNA/K_8/JTS-1 complex. Both 100 and 250 μg/ml DNA were tested in different isotonic solutions to determine if there was any effect on particle size. Mannitol solutions provided the smallest sized particles over time. Particle

size remained similar at one (1) hour after addition to isotonic trehalose, sucrose, lactose, mannitol or NaCl.

The Effects of Tween80 on the 100 μg/ml DNA/K_8/JTS-1 Complex

The following is useful in characterizing the DNA/K_8/JTS-1 complex. The effects of Tween80 on particle size was examined. The complex was dissolved in water for injection ("WFI") and 5% mannitol. Tween80 did not enhance particle size.

The Effects of Filtration of Peptide-DNA Complexes on Particle Size

The following is useful in characterizing the DNA/K_8/JTS-1 complex. The effects of filtration of peptide-DNA complexes on particle size were studied. DNA preparation in the range of 100-500 μg/ml were used. Results show large particles still exist after filtration of the K_8/DNA/JTS-1 complex.

The Effects of Successive Addition and Filtration of K_8/DNA/JTS-1 Complexes

The following is useful in characterizing the DNA/K_8/JTS-1 complex. The effects of successive addition and filtration of K_8/DNA/JTS-1 complexes were examined. DNA preparations in 100 μg/ml increments were tested. Small particles (<200 nm) were obtained but a solubility limit in the amount of DNA and peptide material in solution exists. All particles appear to be stable and suitable for injection.

The Effects of Centrifugation of Aggregates to Obtain Small Complexes of DNA/K_8/JTS-1

The following is useful in characterizing the DNA/K_8/JTS-1 complex. The effect of centrifugation of aggregates was examined. Particle size can be reduced by

using centrifugation at selective speeds ranging from 11,000 to 14,000 rpm.

The Effects of JTS-1 Concentration on DNA/K_8/JTS-1 Particle Size and on Transfection Efficiency

The following is useful in characterizing the DNA/K_8/JTS-1 complex. The concentration of JTS-1 was altered to examine the effects on particle size and transfection efficiency in myotubes. Particle size was measured at 400 μg/ml DNA in 5% mannitol, or at 100 μg/ml DNA in water. It is apparent from these experiments that particle size increases significantly once the JTS-1/DNA ratio is greater than 0.3.

DNA transfection at different JTS-1 ratios was also analyzed. Transfection efficiency decreases if the JTS-1/DNA ratio was < 0.5. These results can be seen in Figure 12.

Transfection of 4Mbr-5 Bronchus Cells with K_6 or K_7 Peptides and JTS-1 Peptide

Figure 13 examines the transfection of 4Mbr-5 (monkey bronchis epithelial) cells using K_6 or K_7 peptides in conjunction with JTS-1. DNA complexes were made by condensing a CMV-β-galactosidase expression vector with transferrin/K_6 or K_7 and unmodified K_6 or K_7 to create a positive particle. The lytic peptides were then added and were bound to the DNA complex through ionic interactions. Transfection experiments using the 4Mbr cells followed the procedures as discussed above. Between 3-12 μgs of DNA complex was incubated with $3x10^5$ 4Mbr cells. After 24 hours, the cells were stained with X-gal for β-galactosidase expression (see above). Up to 50% of the cells were positively stained blue where 9 μg of DNA complex with K_7 was used, as compared to 40% using K_6. In the absence of peptides, no cells were stained blue.

Cell Transfections of C_2C_{12} Muscle Cells

Additional studies using C_2C_{12} muscle cells consider the effects of charge ratios and serum additions, as well as other factors, on transfection. Using the above procedures, C_2C_{12} cells were transfected with DNA/K_8/JTS-1 complexes, using a 1/3/1 ratio. Up to 60% of the cells were positively stained blue using 20 mg of DNA complex. In the absence of peptides, no cells were stained blue. When the ratios were changed from 1/3/1 to 1/6/1 transfection rates dropped 2-fold. Transfection rates dropped as much as 75% when serum was added to the assay using 10 mg of DNA per well and the charge ratio was changed from 1/3/1 to 1/6/1; with 20 mg of DNA under the same parameters, transfection rates dropped by 40%; 90% using 30 mg of DNA; and 80% using 40 mg of DNA complex. DNA complexed with lipofectamine showed no transfection rates at all. Lipofectamine was used as a control.

Transfection of RAW264 Cells Using K_8/JTS-1/DNA

The effects of DNA/K_8/JTS-1 complexes on DNA transfection in macrophage (RAW264) cells were studied, as well as transfection of K_8/JTS-1/DNA complexes in synovialcytes (HIG82). The same transfection procedures as discussed above were used.

With RAW264 cells, DNA transfection efficiency can be correlated at least qualitatively with the following parameters: (1) the presence of the binding molecule, *i.e.*, the condensing component; (2) the presence of a fusogenic component; and (3) DNA dose.

DNA/K_8/JTS-1 Mediated Gene Delivery Into HepG2 Cells

DNA/K_8/JTS-1 complexes were associated noncovalently by ionic interaction as described above. This procedure allowed the addition of more membrane active peptide per DNA complex. The different ratios of DNA, K_8 and JTS-1 were calculated by considering the negative charges of DNA (phosphate groups) and JTS-1 (carboxylic groups), and the

positive charges of K_8 (ϵ-amino groups). DNA was mixed with K_8 at a phosphate to amino group ratio of 1:2, 1:3 and 1:4. Next, JTS-1 was added to form positively, neutral or negatively charged DNA/K_8/JTS-1 complexes. These complexes had the tendency to form microaggregates slowly over time. To determine if these complexes would allow gene expression in HepG2 cells, the *Photinus pyralis* luciferase gene under the control of the early cytomegalovirus ("CMV") enhancer and promoter was used as a reporter gene. Cells were incubated with DNA/K_8/JTS-1 complexes containing increasing amounts of K_8 and JTS-1. Twenty-four hours after gene delivery, the cells were harvested and cellular extracts were analyzed for luciferase activity.

When cells were incubated with DNA or DNA and JTS-1, no significant increase of luciferase activity was observed. Incubating cells with DNA/K_8 complexes led to a 50- to 100-fold increase of luciferase activity. When DNA/K_8/JTS-1 complexes were incubated with cells, a dramatic increase of gene expression was observed. Maximal gene expression was at least 100,000-fold over background and was achieved with positively charged and neutral DNA/K_8/JTS-1 complexes having a phosphate to amino group ratio of 1:3 or 1:4. When cells were incubated with DNA or DNA and maximal amounts of JTS-1, no increase in gene expression over background was observed. Moreover, incubating cells with DNA/K_8 complexes led only to a 50- to 100-fold increase of luciferase expression. Thus, efficient gene expression was obtained using K_8, as well as JTS-1.

Gene transfer was independent of a receptor ligand, *i.e.*, receptor ligand. For high levels of gene expression no receptor ligand was necessary. Since under all conditions tested, positively charged and neutral DNA/K_8/JTS-1 complexes led to 2- to 4-fold higher levels of gene expression than negatively charged complexes, it is likely that cell binding ionic interaction at the cell surface is important. This is similar to DNA/cationic

liposome complexes, which bind to anionic groups on the cell surface and enter the cell by phagocytosis or other unknown mechanism. Receptor independent gene expression has been also reported for DNA/poly-L-lysine/influenza peptide complexes. The presence of a ligand like transferrin only made a 1.5- to 8-fold difference in gene expression. Moreover, the effect of the ligand was cell type dependent.

To investigate the correlation between DNA in complex form and level of gene expression, a dose response curve was performed. $1x10^5$ HepG2 cells were incubated with 0, 1, 3, 6, 9, 12, 15, 18 μg of DNA in complex form and 24 hours after gene delivery luciferase activity was determined. Incubation of cells with increasing amounts of DNA produced a non-linear response of gene expression. With 1 μg of DNA $1x10^3$ light units/mg protein were achieved, with 3 μg of DNA $1x10^7$ light units/mg protein were achieved, and with 6 to 15 μg of DNA $1x10^8$ light units/mg protein were achieved. In fact, with 6 to 9 μg of DNA maximal levels of gene expression was achieved. No further increase was observed for higher DNA amounts. This lack of increase was not due to cytopathic effects of DNA/K_8/JTS-1 complexes, since the morphology of the cells was unchanged and the protein concentration of cell homogenates did not decrease significantly. Therefore, there is a threshold, which has to be overcome to achieve high levels of gene expression. It is possible that a critical number of membrane active peptides have to be present in an endosomal compartment to mediate DNA release into the cytoplasm.

To determine what percentage of cells expressed the reporter gene after gene delivery, a plasmid containing a CMV-*E. coli* β-galactosidase expression cassette was used. Cells were incubated with positive DNA/K_8/JTS-1 complexes and 24 hours after the gene delivery the cells were stained with X-gal. Twenty-five to 30% of cells were

positive for β-galactosidase expression. In contrast, no blue cells were observed in control cells.

Comparison of Gene Transfer Activity of DNA/K_8/JTS-1 Complexes With DNA/K_8/INF-7 Complexes and Recombinant Adenoviral Vectors

JTS-1 mediated gene delivery for DNA/K_8 complexes was compared with the gene transfer activity of INF-7. Positively, neutral and negatively charged complexes were formed similar to DNA/K_8/JTS-1 complexes and 24 hours after gene delivery luciferase activity was determined. For every DNA/K_8/INF-7 complex condition tested, the achieved level of gene expression was at least 1000-fold lower in direct comparison to DNA/K_8/JTS-1 complexes. This difference in gene transfer activity corresponded well to the observed differences in the hemolytic activity of JTS-1 and INF-7 using the hemolysis assay protocol described herein. However, the membrane activity of peptides is not the only factor which determines gene transfer activity. Single amino acid substitutions in the JTS-1 sequence which do not affect the membrane activity of the peptide will lead to considerable differences in gene transfer activity.

To compare the novel DNA/K_8/JTS-1 complexes with a known viral gene delivery system, HepG2 cells were infected with a recombinant adenovirus containing the same CMV-luciferase expression cassette (Adv/CMV-luc) as the plasmid. The adenovirus was grown in 293 cells and purified by double banding on CsCl gradients. The concentration of the virus was determined by ultraviolet spectrophotometric analysis and plaque assay, and the virus was stored in 10% (v/v) glycerol at -70°C. Adenovirus was thawed and used immediately for experiments.

HepG2 cells were incubated with increasing M.O.I. of Adv/CMV-luc. Twenty-four hours after infection the cells were harvested and luciferase activity was determined.

There was a linear increase in gene expression from a M.O.I. of 0.1 to 100. At an M.O.I. of 1000 no further increase was observed, due to cytopathic effects of the recombinant adenovirus. The maximal achieved level of gene expression was around 10^9 light units/mg protein. This was 10- to 50-fold higher than gene expression achieved with DNA/K_8/JTS-1 complexes. This result indicates the potency of DNA/K_8/JTS-1 complexes for the use of gene transfer in cultured cells. The observed difference between the recombinant adenovirus and DNA/K_8/JTS-1 complexes can be due to a number of reasons. For example, after entry of adenovirus particles into the cytoplasm, they are translocated to the nuclear pore for efficient viral DNA delivery into the nucleus. This finding could be significant, since the incorporation of a nuclear localization sequence into DNA vectors increased gene expression 5- to 10-fold.

DNA/K_8/JTS-1 Mediated Gene Delivery Into Mammalian Cells

Cell lines from different species and organs were tested. Cells were incubated with positive DNA/K_8/JTS-1 complexes as prepared above using the *E. coli* β-galactosidase as a reporter gene (see description above). Twenty-four hours after gene delivery cells were stained with X-gal and the percentage of blue cells was determined. The transfection efficiencies in 14 cell lines varied between 1 and 50% with a mean of 23%. These results indicate that DNA/K_8/JTS-1 complexes can be used to transduce a broad range of cell lines *in vitro*. However, the efficiency varies from cell line to cell line as observed with other non-viral and viral delivery systems. For receptor dependent gene delivery, the transduction efficiency of cells correlates well with expression levels of the specific receptor. For receptor independent gene delivery the basic mechanism for cell type variation is poorly understood, but has been documented, especially for DNA/cationic liposome complexes. The type of cells tested

included fibroblast, glioma, myoblast, colon carcinoma, hepatoma, ovarian cancer and embryonic kidney. Cell lines tested included 3T3, Watanabe, 9L, C6, C_2C_{12}, Sol8, MCA-26, HCT-116, ML3, HepG2, Skov3 and 293.

DNA/K_8/JTS-1 Mediated Delivery of the Rabbit LDL-Receptor Gene Into Watanabe Fibroblasts

LDL-receptor ("LDL-R") deficiency is one of the most devastating lipid disorders leading to coronary atherosclerosis and myocardial infarction. Recombinant adenoviral vectors containing the LDL-R gene have been used to transiently correct the cholesterol levels in two animal models for hypercholesterolemia. To access if non-viral vectors can be utilized to deliver the LDL-R into cells, Watanabe fibroblasts were incubated with DNA/K_8/JTS-1 complexes. Watanabe fibroblasts were derived from skin biopsies of Watanabe rabbits, which bear an inframe deletion of 12 nucleotides that eliminate four amino acids from the cysteine-rich ligand binding domain of the LDL-R. This deletion prevents LDL-R mediated uptake and degradation of LDL particles, resulting in dramatic increases of plasma cholesterol levels. The plasmid CMV-rbLDL-R containing the rabbit LDL receptor was constructed by digestion of the plasmid pAdL1/RSV-rbLDL-R with Xba I and Hind III. The isolated fragment was cloned into the plasmid pcDNA3, which contains a CMV expression cassette.

DNA/K_8/JTS-1 complexes were used to deliver the rabbit LDL-R ("rbLDL-R") gene under the control of the CMV enhancer and promoter element into Watanabe fibroblasts. Twenty-four hours after gene delivery the cells were incubated with [^{125}I]-labeled human LDL for five hours and LDL binding/uptake and degradation was determined. Studies were performed in the absence and presence of 200-fold excess unlabeled LDL. In comparison to control cells, there was a 4- to 5-fold increase of specific binding and uptake of LDL in Watanabe fibroblast after rbLDL-R gene/K_8/JTS-1 mediated gene delivery. Furthermore,

a 2-fold increase of LDL degradation was observed, indicating that the DNA/K_8/JTS-1 complexes can be utilized to express functional therapeutic genes. This level of LDL receptor replacement is low in comparison to adenovirus mediated LDL receptor replacement in hepatocytes of LDL deficient mice or rabbits. However, it demonstrates that DNA/peptide complexes can correct metabolic diseases.

Targeting/DNA/K_8/JTS-1 Complexes

Figures 14-18 set forth various surface ligands that can be coupled to binding molecules, such as K_8, or coupled to JTS-1 to direct delivery of the nucleic acid to a specific cell, see below. For delivery to hepatocytes, peptides containing carbohydrates for uptake via the asialoglycoprotein receptor were constructed (Figures 14 and 15). For delivery to cells with mannose or mannose-6-phosphate receptors, ligands in Figure 16 were coupled to JTS-1 or K_8. The following is a list of other receptor ligands coupled to K_8 or JTS-1 that have also been constructed and characterized.

Condensing Peptides	Molecular Weight	Parent Ions
FolateK8 FOLATECGSGSGSGSGYKAKKKKKK-KKWK	---	---
ERPJTS1 SHLRKLRKRLLRAASLFESLLELLESL-WELLLEA	4048	4044.3
CS35K8 EWSPCSVTCGNGIQVRIKPGSGSGSGS-GSGYKAKKKKKKKKWK	4554.6	4554.5
SPDPK8 SPDPGSGSGSGSGSGYKAKKKKKKKKWK	---	---
Man-6-PO4K8 Man-6-PO4-SCGSGSGSGSGSGYKAK-KKKKKKKWK	2990	2989.6

In addition to the above, RGD targeting ligands can also be attached to K_8 peptides as set forth in Figure 17. Such a ligand is useful in delivery of therapeutic genes to connective tissue, wounds, and for healing. Likewise, the lipids in Figure 18 can be used for delivery to hepatocytes.

Cell Transformation

One embodiment of the present invention includes cells transformed with nucleic acid associated with the nucleic acid transporter systems described above. Once the cells are transformed, the cells will express the protein, polypeptide or RNA encoded for by the nucleic acid. Cells included, but are not limited to, liver, muscle and skin. This is not intended to be limiting in any manner.

The nucleic acid which contains the genetic material of interest is positionally and sequentially oriented within the host or vectors such that the nucleic acid can be transcribed into RNA and, when necessary, be translated into proteins or polypeptides in the transformed cells. A variety of proteins and polypeptides can be expressed by the sequence in the nucleic acid cassette in the transformed cells. These products may function as intracellular or extracellular structural elements, ligands, hormones, neurotransmitters, growth regulating factors, apolipoproteins, enzymes, serum proteins, receptors, carriers for small molecular weight compounds, drugs, immunomodulators, oncogenes, tumor suppressors, toxins, tumor antigens, antigens, antisense inhibitors, triple strand forming inhibitors, ribozymes, or as a ligand recognizing specific structural determinants on cellular structures for the purpose of modifying their activity.

Transformation can be done either by *in vivo* or *ex vivo* techniques. One skilled in the art will be familiar with such techniques for transformation. Transformation by *ex vivo* techniques includes co-transfecting the cells

with DNA containing a selectable marker. This selectable marker is used to select those cells which have become transformed. Selectable markers are well known to those who are skilled in the art.

For example, one approach to gene therapy for hepatic diseases is to remove hepatocytes from an affected individual, genetically alter them *in vitro*, and reimplant them into a receptive locus. The *ex vivo* approach includes the steps of harvesting hepatocytes, cultivating the hepatocytes, transducing or transfecting the hepatocytes, and introducing the transfected hepatocytes into the affected individual.

The hepatocytes may be obtained in a variety of ways. They may be taken from the individual who is to be later injected with the hepatocytes that have been transformed or they can be collected from other sources, transformed and then injected into the individual of interest.

Once the *ex vivo* hepatocyte is collected, it may be transformed by contacting the hepatocytes with media containing the nucleic acid transporter and maintaining the cultured hepatocytes in the media for sufficient time and under conditions appropriate for uptake and transformation of the hepatocytes. The hepatocytes may then be introduced into an orthotopic location (the body of the liver or the portal vasculature) or heterotopic locations by injection of cell suspensions into tissues. One skilled in the art will recognize that the cell suspension may contain: salts, buffers or nutrients to maintain viability of the cells; proteins to ensure cell stability; and factors to promote angiogenesis and growth of the implanted cells.

In an alternative method, harvested hepatocytes may be grown *ex vivo* on a matrix consisting of plastics, fibers or gelatinous materials which may be surgically implanted in an orthotopic or heterotopic location after transduction. This matrix may be impregnated with factors to promote angiogenesis and growth of the implanted cells.

Cells can then be reimplanted. The above are only examples and are nonlimiting.

Administration

Administration as used herein refers to the route of introduction of the nucleic acid transporters into the body. Administration includes intravenous, intramuscular, topical, or oral methods of delivery. Administration can be directly to a target tissue or through systemic delivery.

In particular, the present invention can be used for administering nucleic acid for expression of specific nucleic acid sequence in cells. Routes of administration include intramuscular, aerosol, olfactory, oral, topical, systemic, ocular, intraperitoneal and/or intratracheal. A preferred method of administering nucleic acid transporters is by intravenous delivery. Another preferred method of administration is by direct injection into the cells.

Transfer of genes directly has been very effective. Experiments show that administration by direct injection of DNA into joints and thyroid tissue results in expression of the gene in the area of injection. Injection of plasmids containing IL-1 into the spaces of the joints results in expression of the gene for prolonged periods of time. The injected DNA appears to persist in an unintegrated extrachromosomal state. This means of transfer is one of the preferred embodiments.

In addition, another means to administer the nucleic acid transporters of the present invention is by using a dry powder form for inhalation. One compound which can be used is polyvinylpyrrolidone ("PVP"), an amorphous powder. PVP is a polyamide that forms complexes with a wide variety of substances and is chemically and physiologically inert. Specific examples of suitable PVP's are Plasdone-C®15, MW 10,000 and Plasdone-C®30, MW 50,000. Furthermore, administration may also be through

an aerosol composition or liquid form into a nebulizer mist and thereby inhaled.

The special delivery route of any selected vector construct will depend on the particular use for the nucleic acid associated with the nucleic acid transporter. In general, a specific delivery program for each nucleic acid transporter used will focus on uptake with regard to the particular targeted tissue, followed by demonstration of efficacy. Uptake studies will include uptake assays to evaluate cellular uptake of the nucleic acid and expression of the specific nucleic acid of choice. Such assays will also determine the localization of the target nucleic acid after uptake, and establishing the requirements for maintenance of steady-state concentrations of expressed protein. Efficacy and cytotoxicity is then tested. Toxicity will not only include cell viability but also cell function.

Incorporated DNA into transporters, as described herein, which undergo endocytosis increases the range of cell types that will take up foreign genes from the extracellular space.

The chosen method of delivery should result in cytoplasmic accumulation and optimal dosing. The dosage will depend upon the disease and the route of administration but should be between 1-1000 mg/kg of body weight/day. This level is readily determinable by standard methods. It could be more or less depending on the optimal dosing. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend upon disease delivery vehicle and efficacy data from clinical trials.

Establishment of therapeutic levels of DNA within the cell is dependent upon the rate of uptake and degradation. Decreasing the degree of degradation will prolong the intracellular half-life of the DNA.

Methods of Use

Direct DNA Delivery to Muscle

The muscular dystrophies are a group of diseases that result in abnormal muscle development, due to many different reasons. These diseases can be treated by using the direct delivery of genes with the nucleic acid transporters of the present invention resulting in the production of normal gene product. Delivery to the muscle using the present invention is done to present genes that produce various antigens for vaccines against a multitude of infections of both viral and parasitic origin. The detrimental effects caused by aging can also be treated using the nucleic acid delivery system described herein. Since the injection of the growth hormone protein promotes growth and proliferation of muscle tissue, the growth hormone gene can be delivered to muscle, resulting in both muscle growth and development, which is decreased during the later portions of the aging process. Genes expressing other growth related factors can be delivered, such as Insulin Like Growth Factor-1 (IGF-1). Furthermore, any number of different genes may be delivered by this method to the muscle tissue.

IGF-1 can be used to deliver DNA to muscle, since it undergoes uptake into cells by receptor-mediated endocytosis. This polypeptide is 70 amino acids in length and is a member of the growth promoting polypeptides structurally related to insulin. It is involved in the regulation of tissue growth and cellular differentiation affecting the proliferation and metabolic activities of a wide variety of cell types, since the polypeptide has receptors on many types of tissue. As a result, the nucleic acid transporter delivery system of the present invention utilizes IGF-1 as a ligand for tissue-specific nucleic acid delivery to muscle. The advantage of the IGF-1/nucleic acid delivery system is that the specificity and the efficiency of the delivery is greatly increased due to a great number of cells coming into contact with the

ligand/nucleic acid complex with uptake through receptor-mediated endocytosis. Using the nucleic acid described above in the delivery systems of the present invention with the use of specific ligands for the delivery of nucleic acid to muscle cells provides treatment of diseases and abnormalities that affect muscle tissues.

In addition to the above, Factor IX can also be delivered to the muscle cells. DNA encoding Factor IX can be delivered using the nucleic acid transporters of the present invention. As a result, the nucleic acid transporter delivery system of the present invention utilizes nucleic acids encoding Factor IX to treat cells which are Factor IX deficient and are susceptible to disease and abnormalities due to such a deficiency. DNA encoding Factor IX can be coupled or associated with K_8 and JTS-1 as described above. The complex can then be delivered directly to muscle cells for expression. The preferred ratio of DNA to K_8 to JTS-1 is 1:3:1. Direct injection of the above complex is preferred. Use of the above nucleic acid delivery system of the present invention for the delivery of nucleic acid expressing Factor IX to muscle cells provides treatment of diseases and abnormalities that affect muscle tissues.

Direct DNA Delivery to Osteogenic Cells

There are many other problems that occur during the aging process, but one major problem is osteoporosis, which is the decrease in overall bone mass and strength. The direct nucleic acid delivery system of the present invention can be used to deliver genes to cells that promote bone growth. The osteoblasts are the main bone forming cell in the body, but there are other cells that are capable of aiding in bone formation. The stromal cells of the bone marrow are the source of stem cells for osteoblasts. The stromal cells differentiate into a population of cells known as Inducible Osteoprogenitor Cells (IOPC), which then under induction of growth

factors, differentiate into Determined Osteoprogenitor Cells (DOPC). It is this population of cells that mature directly into bone producing cells. The IOPCs are also found in muscle and soft connective tissues. Another cell involved in the bone formation process is the cartilage-producing cell known as the chondrocyte.

The factor that has been identified to be involved in stimulating the IOPCs to differentiate is known as Bone Morphogenetic Protein (BMP). This 19,000 MW protein was first identified from demineralized bone. Another factor similar to BMP is Cartilage Induction Factor (CIF), which functions to stimulate IOPCs to differentiate also, starting the pathway of cartilage formation, cartilage calcification, vascular invasion, resorption of calcified cartilage, and finally induction of new bone formation. Cartilage Induction Factor has been identified as being homologous to Transforming Growth Factor β.

Since osteoblasts are involved in bone production, genes that enhance osteoblast activity can be delivered directly to these cells. Genes can also be delivered to the IOPCs and the chondrocytes, which can differentiate into osteoblasts, leading to bone formation. BMP and CIF are the ligands that can be used to deliver genes to these cells. Genes delivered to these cells promote bone formation or the proliferation of osteoblasts. The polypeptide, IGF-1 stimulates growth in hypophysectomized rats which could be due to specific uptake of the polypeptide by osteoblasts or by the interaction of the polypeptide with chondrocytes, which result in the formation of osteoblasts. Other specific bone cell and growth factors can be used through the interaction with various cells involved in bone formation to promote osteogenesis.

Nonlimiting examples of genes expressing the following growth factors which can be delivered to these cell types are Insulin, Insulin-Like Growth Factor-1, Insulin-Like Growth Factor-2, Epidermal Growth Factor, Transforming Growth Factor-α, Transforming Growth Factor-

β, Platelet Derived Growth Factor, Acidic Fibroblast Growth Factor, Basic Fibroblast Growth Factor, Bone Derived Growth Factors, Bone Morphogenetic Protein, Cartilage Induction Factor, Estradiol, and Growth Hormone. All of these factors have a positive effect on the proliferation of osteoblasts, the related stem cells, and chondrocytes. As a result, BMP or CIF can be used as conjugates to deliver genes that express these growth factors to the target cells by the intravenous injection of the nucleic acid/Protein complexes of the present invention. Using the nucleic acid described above in the delivery systems of the present invention with the use of specific ligands for the delivery of nucleic acid to bone cells provides treatment of diseases and abnormalities that affect bone tissues.

Direct DNA Delivery to the Synovialcytes

The inflammatory attack on joints in animal models and human diseases may be mediated, in part, by secretion of cytokines such as IL-1 and IL-6 which stimulate the local inflammatory response. The inflammatory reaction may be modified by local secretion of soluble fragments of the receptors for these ligands. The complex between the ligand and the soluble receptor prevents the ligand from binding to the receptor which is normally resident on the surface of cells, thus preventing the stimulation of the inflammatory effect. Therapy consists of the construction of a vector containing the soluble form of receptors for appropriate cytokines (for example, IL-1), together with promoters capable of inducing high level expression in structures of the joint and a formulation which enables efficient uptake of this vector. This DNA is then used with the DNA transporters of the present invention. This DNA is injected into affected joints where the secretion of an inhibitor for IL-1 such as a soluble IL-1 receptor or natural IL-I inhibitor modifies the local inflammatory response and resulting arthritis.

This method is useful in treating episodes of arthritis which characterize many "autoimmune" or "collagen vascular" diseases. This method can also prevent disabling injury of large joints by inflammatory arthritis.

In addition to the above, the present invention can also be used with the following method. Current therapy for severe arthritis involves the administration of pharmacological agents including steroids to depress the inflammatory response. Steroids can be administered systemically or locally by direct injection into the joint space.

Steroids normally function by binding to receptors within the cytoplasm of cells. Formation of the steroid-receptor complex changes the structure of the receptor so that it becomes capable of translocating to the nucleus and binding to specific sequences within the genome of the cell and altering the expression of specific genes. Genetic modifications of the steroid receptor can be made which enable this receptor to bind naturally occurring steroids with higher affinity, or bind non-natural, synthetic steroids, such as RU486. Other modifications can be made to create steroid receptor which is "constitutively active" meaning that it is capable of binding to DNA and regulating gene expression in the absence of steroid in the same way that the natural steroid receptor regulates gene expression after treatment with natural or synthetic steroids.

Of particular importance is the effect of glucocorticoid steroids such as cortisone, hydrocortisone, prednisone, or dexamethasone which are the most important drugs available for the treatment of arthritis. One approach to treating arthritis is to introduce a vector in which the nucleic acid cassette expresses a genetically modified steroid receptor into cells of the joint, *e.g.*, a genetically modified steroid receptor which mimics the effect of glucocorticoids but does not require the presence of glucocorticoids for effect. This is termed

the glucocortico-mimetic receptor. This is achieved by expression of a constitutively active steroid receptor within cells of the joint which contains the DNA binding domain of a glucocorticoid receptor. This induces the therapeutic effects of steroids without the systemic toxicity of these drugs. Alternatively, steroid receptors which have a higher affinity for natural or synthetic glucocorticoids, such as RU486, can be introduced into the joint. These receptors exert an increased anti-inflammatory effect when stimulated by non-toxic concentrations of steroids or lower doses of pharmacologically administered steroids. Alternatively, constitution of a steroid receptor which is activated by a novel, normally-inert steroid enables the use of drugs which would affect only cells taking up this receptor. These strategies obtain a therapeutic effect from steroids on arthritis without the profound systemic complications associated with these drugs. Of particular importance is the ability to target these genes differentially to specific cell types (for example synovial cells versus lymphocytes) to affect the activity of these cells.

As described in U.S. Patent No. 5,364,791 to Vegeto, et al., entitled "Progesterone Receptor Having C Terminal Hormone Binding Domain Truncations," and U.S. Application, Serial No. 07/939,246, entitled "Mutated Steroid Hormone Receptors, Methods for Their Use and Molecular Switch for Gene Therapy," Vegeto, et al., filed September 2, 1992, both hereby incorporated by reference (including drawings), genetically modified receptors, such as the glucocortico-mimetic receptor, can be used to create novel steroid receptors including those with glucocortico-mimetic activity. The steroid receptor family of gene regulatory proteins is an ideal set of such molecules. These proteins are ligand activated transcription factors whose ligands can range from steroids to retinoids, fatty acids, vitamins, thyroid hormones and other presently unidentified small molecules. These compounds bind to

receptors and either up-regulate or down-regulate transcription.

The preferred receptor of the present invention is modification of the glucocorticoid receptor, *i.e.*, the glucocorticoid-mimetic receptor. These receptors can be modified to allow them to bind various ligands whose structure differs from naturally occurring ligands, *e.g.*, RU486. For example, small C-terminal alterations in amino acid sequence, including truncation, result in altered affinity and altered function of the ligand. By screening receptor mutants, receptors can be customized to respond to ligands which do not activate the host cells own receptors.

A person having ordinary skill in the art will recognize, however, that various mutations, for example, a shorter deletion of carboxy terminal amino acids, will be necessary to create useful mutants of certain steroid hormone receptor proteins. Steroid hormone receptors which may be mutated are any of those receptors which comprise the steroid hormone receptor super family, such as receptors including the estrogen, progesterone, glucocorticoid-α, glucocorticoid-β, mineral corticoid, androgen, thyroid hormone, retinoic acid, and Vitamin B3 receptors. Furthermore, DNA encoding for other mutated steroids such as those which are capable of only transrepression or of only transactivation are also within the scope of the above embodiment. Such steroids could be capable of responding to RU486 in order to activate transrepression.

In addition to the above, the present invention can also be used with the following method. Drugs which inhibit the enzyme prostaglandin synthase are important agents in the treatment of arthritis. This is due, in part, to the important role of certain prostaglandin in stimulating the local immune response. Salicylates are widely used drugs but can be administered in limited doses which are often inadequate for severe forms of arthritis.

Gene transfer using the present invention is used to inhibit the action of prostaglandin synthase specifically in affected joints by the expression of an antisense RNA for prostaglandin synthase. The complex formed between the antisense RNA and mRNA for prostaglandin synthase interferes with the proper processing and translation of this mRNA and lowers the levels of this enzyme in treated cells. Alternatively RNA molecules are used for forming a triple helix in regulatory regions of genes expressing enzymes required for prostaglandin synthesis. Alternatively, RNA molecules are identified which bind the active site of enzymes required for prostaglandin synthesis and inhibit this activity.

Alternatively, genes encoding enzymes which alter prostaglandin metabolism can be transferred into the joint. These have an important anti-inflammatory effect by altering the chemical composition or concentration of inflammatory prostaglandin.

Likewise, the present invention is useful for enhancing repair and regeneration of the joints. The regenerative capacity of the joint is limited by the fact that chondrocytes are not capable of remodelling and repairing cartilaginous tissues such as tendons and cartilage. Further, collagen which is produced in response to injury is of a different type lacking the tensile strength of normal collagen. Further, the injury collagen is not remodeled effectively by available collagenase. In addition, inappropriate expression of certain metalloproteinases is a component in the destruction of the joint.

Gene transfer using promoters specific to chondrocytes (*i.e.*, collagen promoters) is used to express different collagens or appropriate collagenase for the purpose of improving the restoration of function in the joints and prevent scar formation.

Gene transfer for these purposes is affected by direct introduction of DNA into the joint space where it

comes into contact with chondrocytes and synovial cells. Further, the genes permeate into the environment of the joint where they are taken up by fibroblasts, myoblasts, and other constituents of periarticular tissue.

Direct Delivery to the Lungs

Nucleic acid transporters of the present invention can also be used in reversing or arresting the progression of disease involving the lungs, such as lung cancer. One embodiment involves use of intravenous methods of administration to delivery nucleic acid encoding for a necessary molecule to treat disease in the lung. Nucleic acid transporters which express a necessary protein or RNA can be directly injected into the lungs or blood supply so as to travel directly to the lungs. Furthermore, the use of an aerosol or a liquid in a nebulizer mist can also be used to administer the desired nucleic acid to the lungs. Finally, a dry powder form, such as PVP discussed above, can be used to treat disease in the lung. The dry powder form is delivered by inhalation. These treatments can be used to control or suppress lung cancer or other lung diseases by expression of a particular protein encoded by the nucleic acid chosen.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The nucleic acid transporter systems along with the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made

to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specif ically and individually indicated to be incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i)	APPLICANT:	Smith, Louis C. Sparrow, James C. Woo, Savio L.C.
(ii)	TITLE OF INVENTION:	NUCLEIC ACID TRANSPORTERS FOR DELIVERY OF NUCLEIC ACIDS INTO A CELL
(iii)	NUMBER OF SEQUENCES:	39
(iv)	CORRESPONDENCE ADDRESS:	
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	(C) CITY:	Los Angeles
	(D) STATE:	California
	(E) COUNTRY:	U.S.A.
	(F) ZIP:	90071-2066
(v)	COMPUTER READABLE FORM:	
	(A) MEDIUM TYPE:	3.5" Diskette, 1.44 Mb storage
	(B) COMPUTER:	IBM PC compatible
	(C) OPERATING SYSTEM:	IBM MS-DOS (Ver. 5.0)
	(D) SOFTWARE:	WordPerfect (Ver. 5.1)
(vi)	CURRENT APPLICATION DATA:	
	(A) APPLICATION NUMBER:	Not yet assigned
	(B) FILING DATE:	Herewith
	(C) CLASSIFICATION:	
(vii)	PRIOR APPLICATION DATA	
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(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

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(B) REGISTRATION NUMBER: 36,846
(C) REFERENCE/DOCKET NO.: 211/270

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GLFEALLELL ESLWELLLEA 20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GFFEALLELL ESLWELLLEA 20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GLFEALLELL ESWELLLGLF EA 22

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GLFEALLELL EKLWELLLEA 20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

YKAKKKKKKK KWK 13

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GLFEALLELL ESLWELLLE A 21

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GLFEALLELL ESLWELLLEA 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GLFEALLELL ESLWELLLEL YA 22

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GLFEALLELL EELWELLLEA 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GLFEALLELL EELWEALLEA 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GLFEALLELL ESLWELLLEA GGGGC 25

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

SLFEALLELL ESLWELLLEA 20

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GLFEALLELL ESLYELLLEA 20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	20 amino acids
(B) TYPE:	amino acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GLFEALAELL ESLWEALLEA 20

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	20 amino acids
(B) TYPE:	amino acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GLFEALLELL ESPWELLLEA 20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	20 amino acids
(B) TYPE:	amino acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GLFEALLELL ESLWEFLLEA 20

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GLFEAILELL ESLWELLLEA 20

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GLFEALLELW EA 12

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GLFEALLESL WEA 13

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GLFEALLEIL ESLWELLLEA 20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GLFEALLELW EA 12

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GLFEALLELL ESLWEA 16

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GLFEALLELL ESLWEFFLEA 20

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GLFEALLELF ESLWELLEA 19

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	20 amino acids
(B) TYPE:	amino acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GLFESLLELL ESLWELLLEA 20

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	20 amino acids
(B) TYPE:	amino acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GLFEALLELL ESLWELLKEA 20

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	24 amino acids
(B) TYPE:	amino acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GLFEALLELL ESLWELLLEA AEEA 24

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GLFEALLELL ESPWELLLEA GGGSGSGSGS GSGYKAKKKK KKKKWK 46

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(ix) FEATURE:
(D) OTHER INFORMATION: /note= X = ϵ-Pam and attaches to the NH2 of K

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GLFEALLELW EAKX 14

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(ix) FEATURE:
(D) OTHER INFORMATION: /note= X = ϵ-BIOHX and attaches to the NH2 of K

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GLFEALLELL ESLWEAKX 18

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(ix) FEATURE:
(D) OTHER INFORMATION: /note= X = ϵ-BIOHX and attaches to the NH2 of K

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GLFEALLELL ESLWELLLEA KX 22

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(ix) FEATURE:
(D) OTHER INFORMATION: /note= X = ac

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

XGLFEALLEL LESLWELLLE A 21

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(ix) FEATURE:
(D) OTHER INFORMATION: /note= X = Me2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

XGLFEALLEL LESLWELLLE A 21

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

LFEALLELLE SLWELLLEA 19

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	32 amino acids
(B) TYPE:	amino acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GLFEALLELL ESLWEAAAKL SKLEKKLSKL EK 32

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	18 amino acids
(B) TYPE:	amino acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

WEAALAEALA EALAEHLA 18

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	30 amino acids
(B) TYPE:	amino acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

WEAALAEALA EALAEHLAEA LAEALEALAA 30

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(ix) FEATURE:
(D) OTHER INFORMATION: /note= X = Suc

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

XGLFKLLEEW LE 12

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

GLFEAIEGFI ENGWEGMID 19

Claims

1. Nucleic acid transporter system for delivering nucleic acid into a cell, comprising:

a nucleic acid binding complex comprising a binding molecule noncovalently bound to said nucleic acid and associated with a lysis agent.

2. The transporter of claim 1, further comprising a second binding molecule noncovalently bound to said nucleic acid.

3. The transporter of claims 1 or 2, further comprising a plurality of said nucleic acid binding complex or said second binding molecule.

4. The transporter of claims 1 or 2, wherein said binding molecule is K_8.

5. The transporter of claim 1, wherein said lysis agent is JTS-1.

6. The transporter of claims 1 or 5, wherein said lysis agent is attached to said binding molecule by a spacer.

7. A method of using the nucleic acid transporter in claims 1 or 2 for delivering nucleic acid to a cell comprising the steps of contacting said cell with said nucleic acid transporter.

8. A method of treating humans with nucleic acid comprising the steps of administering the nucleic acid transporter in claims 1 or 2, delivery of said nucleic acid to a cell and expression of said nucleic acid in said cell.

9. A cell transformed with the nucleic acid transporter of claims 1 or 2.

10. Nucleic acid transporter for delivering nucleic acid into a cell, comprising:

a first nucleic acid binding complex comprising a first binding molecule noncovalently bound to said nucleic acid and associated with a surface ligand; and

a second nucleic acid binding complex comprising a second binding molecule noncovalently bound to said nucleic acid and associated with a lysis agent.

11. The transporter of claim 10, wherein said lysis agent is JTS-1.

12. The transporter of claim 10, further comprising a third binding molecule noncovalently bound to said nucleic acid.

13. The transporter of claims 10 or 12, further comprising a plurality of said first or said second nucleic acid binding complexes, or said third binding molecule.

14. The transporter of claims 10 or 12, wherein said first, said second, or said third binding molecules are K_8.

15. The transporter of claims 10 or 11, wherein said surface ligand or said lysis agent are associated with their respective binding molecule by a spacer.

16. A method of using the nucleic acid transporter in claims 10 or 12 for delivering nucleic acid or molecules to a cell comprising the steps of contacting said cell with said nucleic acid transporter.

17. A method of treating humans with nucleic acid comprising the steps of administering the nucleic acid transporter in claims 10 or 12, delivery of said nucleic acid to a cell and expression of said nucleic acid in said cell.

18. A cell transformed with the nucleic acid transporter of claims 10 or 12.

19. Nucleic acid transporter system for delivering nucleic acid into a cell, comprising:

a first nucleic acid binding complex comprising a first binding molecule noncovalently bound to said nucleic acid and associated with a surface ligand;

a second nucleic acid binding complex comprising a second binding molecule noncovalently bound to said nucleic acid and associated with a nuclear ligand; and

a third nucleic acid binding complex comprising a third binding molecule noncovalently bound to said nucleic acid and associated with a lysis agent.

20. The transporter of claim 19, wherein said lysis agent is JTS-1.

21. The transporter of claim 19, further comprising a fourth binding molecule noncovalently bound to said nucleic acid.

22. The transporter of claims 19 or 21, further comprising a plurality of said first, said second or said third nucleic acid binding complexes, or said fourth binding molecule.

23. The transporter of claims 19 or 21, wherein said first, said second, said third, or said fourth binding molecules are K_8.

24. The transporter of claims 19 or 20, wherein said surface ligand, said nuclear ligand or said lysis agent are associated with their respective binding molecules by a spacer.

25. A method of using the nucleic acid transporter in claims 19 or 21 for delivering nucleic acid or molecules to a cell comprising the steps of contacting said cell with said nucleic acid transporter.

26. A method of treating humans with nucleic acid comprising the steps of administering the nucleic acid transporter in claims 19 or 21, delivery of said nucleic acid to a cell and expression of said nucleic acid in said cell.

27. A cell transformed with the nucleic acid transporter of claims 19 or 21.

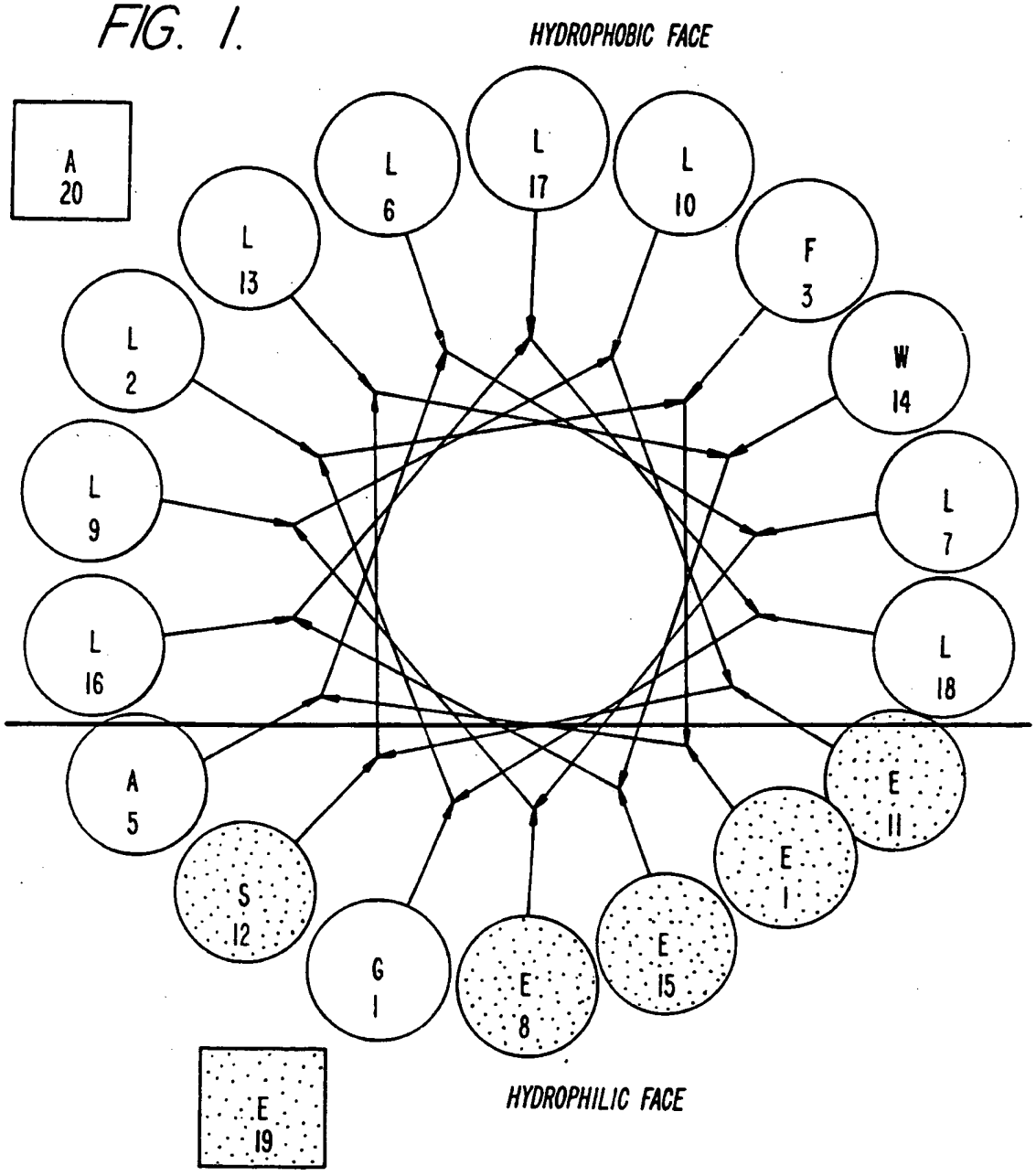
28. JTS-1 or derivative thereof.

29. K_8 or derivative thereof.

30. K_8 or derivative thereof associated with JTS-1 or derivative thereof.

31. A compound of the structure $YKAK_NWK$, wherein n = 1-40, or derivative thereof.

FIG. 1.


HYDROPHOBIC FACE
A 20
L 6
L 17
L 10
L 13
F 3
L 2
W 14
L 9
L 7
L 16
L 18
A 5
E 11
E 1
S 12
G 1
E 8
E 15
E 19
HYDROPHILIC FACE

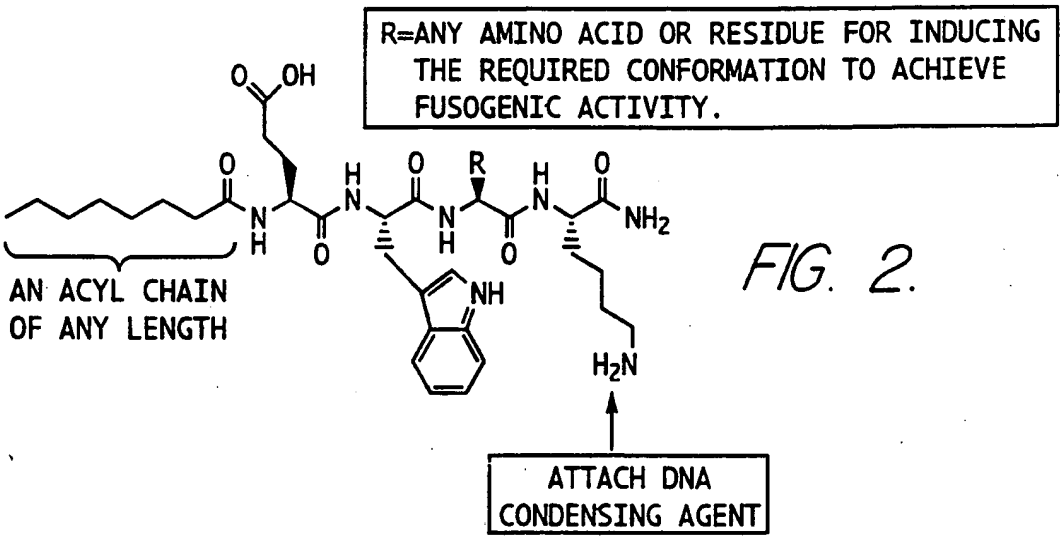


FIG. 2.

FIG. 5.

JTS-1 MEDIATED GENE DELIVERY

CELL LINE	CELL TYPE	PERCENT BLUE CELLS
3T3	FIBROBLAST	30
9L	BRAIN	15
C6	BRAIN	5
MCA-26	COLON	40
HCT-116	COLON	35
ML3	LIVER	5
RAT HEPATOCYTES	PRIMARY	5
SKOV3	OVARY	1
sol 8	MUSCLE	20
L132	LUNG	15
4MBR-5	BRONCHUS	50
293	KIDNEY	90
	HUMAN FIBROBLAST	30
	HUMAN MONOCYTES	2

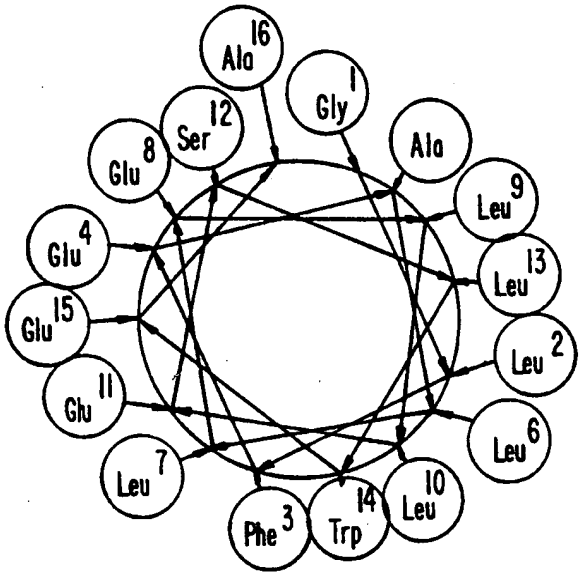


FIG. 3a. I GLFEALLELLESLWEA#

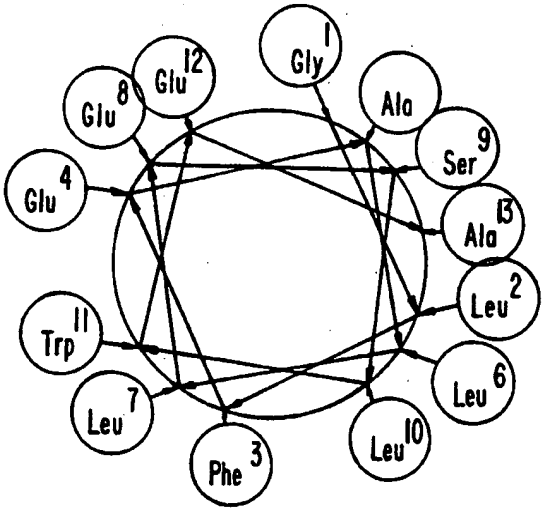
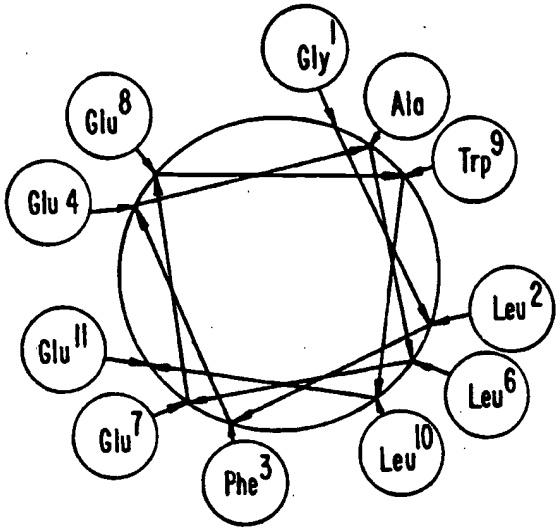
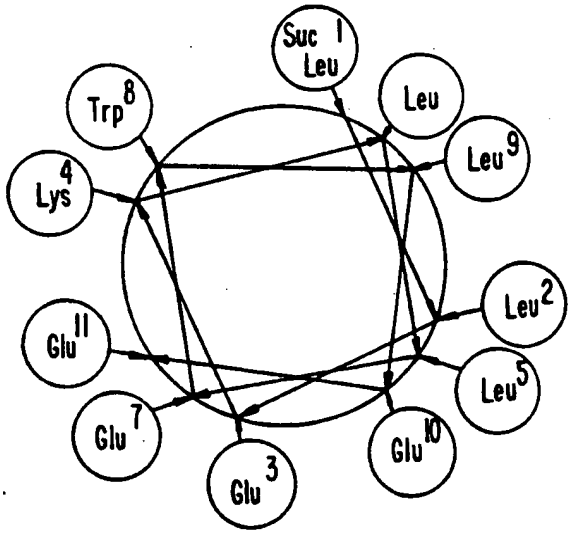


FIG. 3b. II GLFEALLESLWEA#



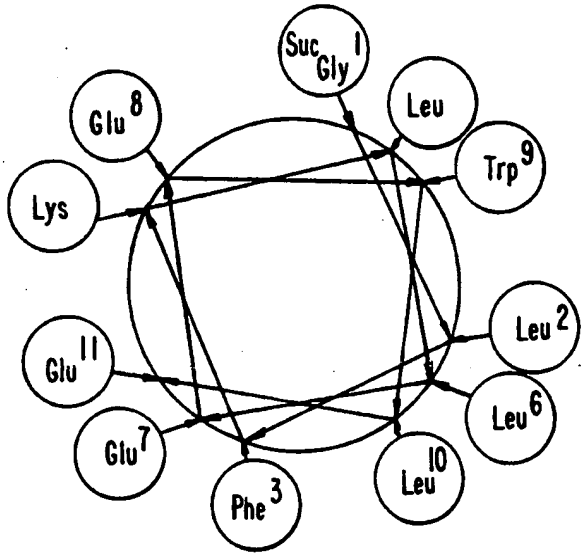
III
GLFEALEEWLE#

FIG. 3c.



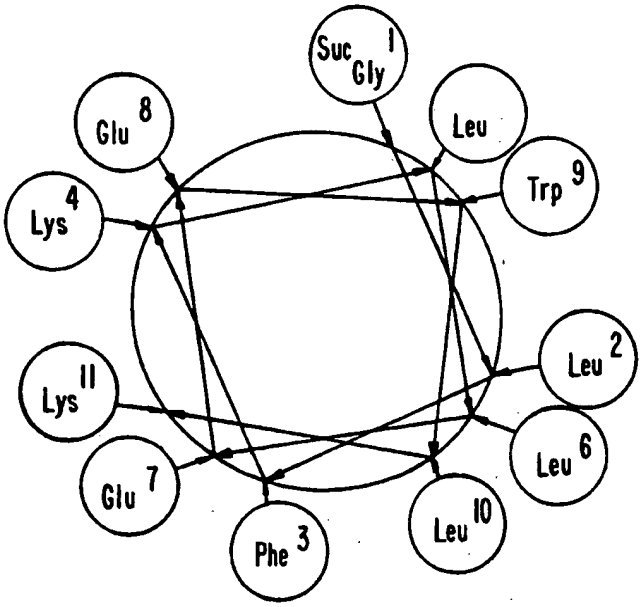
IV
Suc-LLEKLLEWLE #

FIG. 3d.



V
Suc-GLFKLLEEWLE#

FIG. 3e.



VI
Suc-GLFKLLEEWLK#

FIG. 3f.

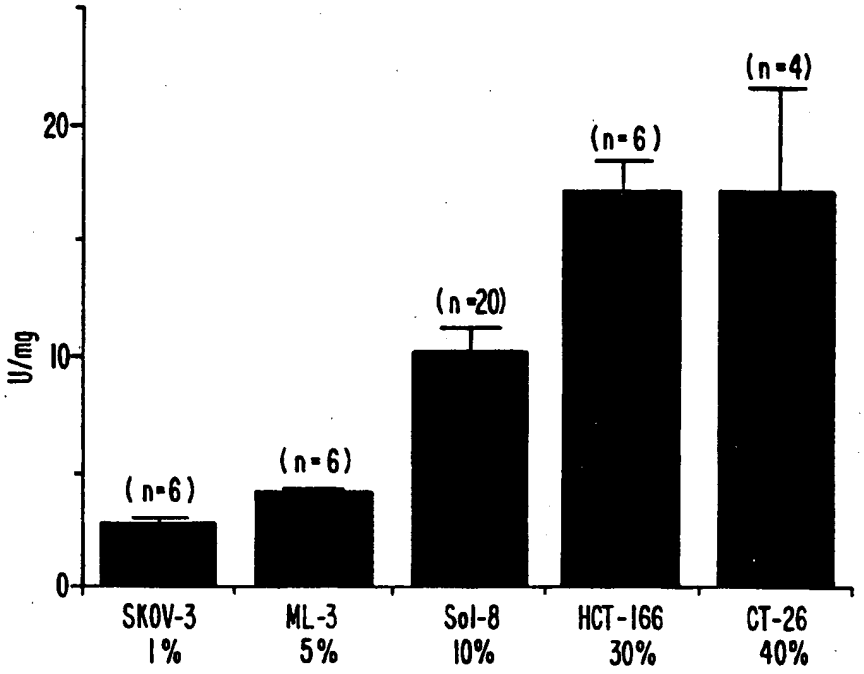


FIG. 4.

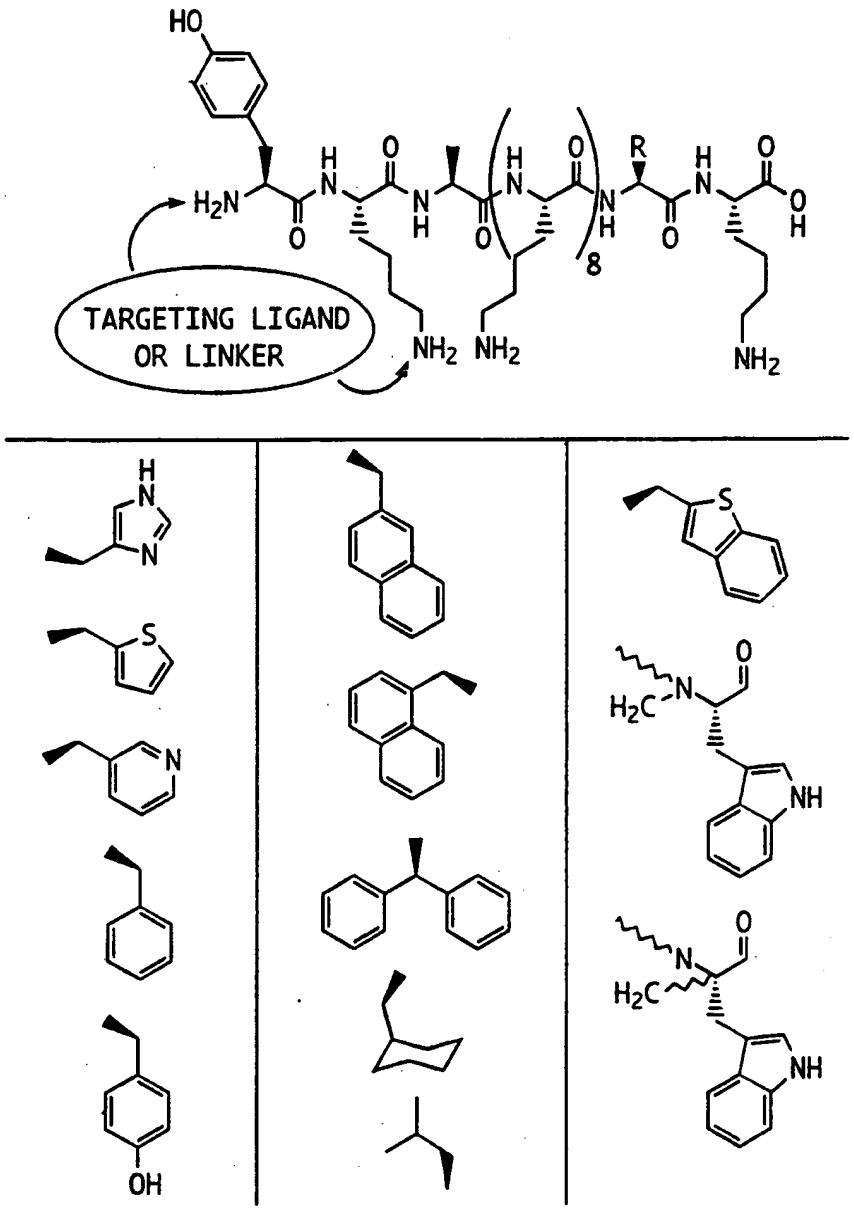


FIG. 6.

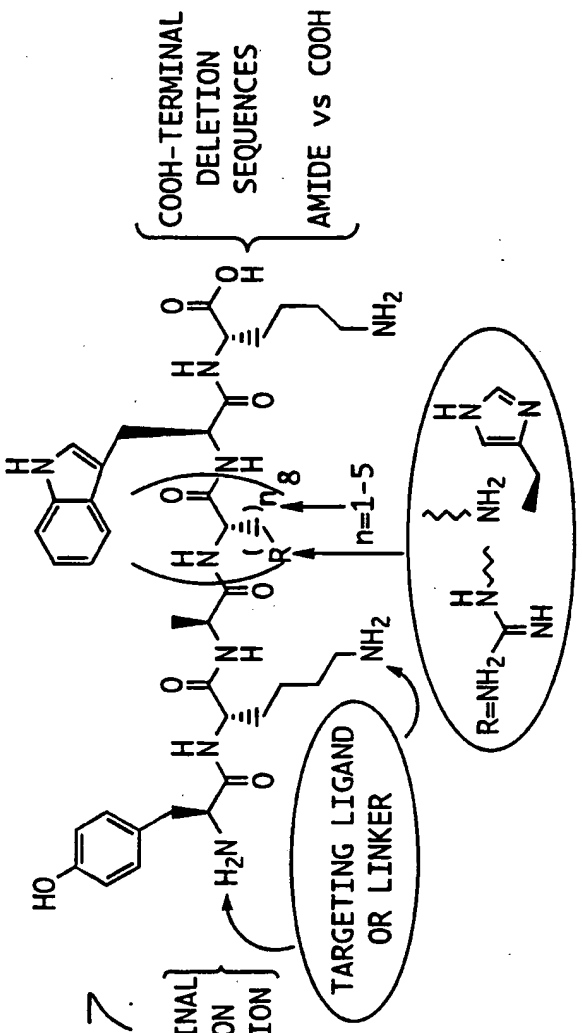

COOH-TERMINAL DELETION SEQUENCES
AMIDE vs COOH
NH2 TERMINAL DELETION ALKYLATION
TARGETING LIGAND OR LINKER
n=1-5
R=NH2

FIG. 7.

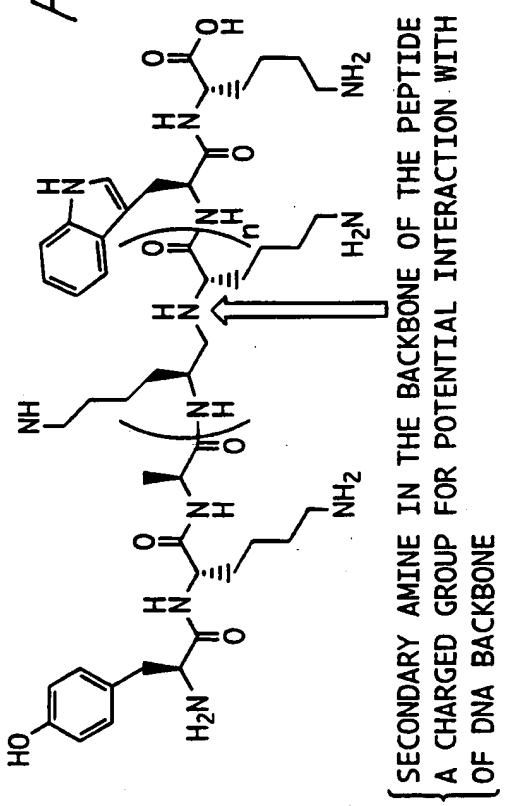
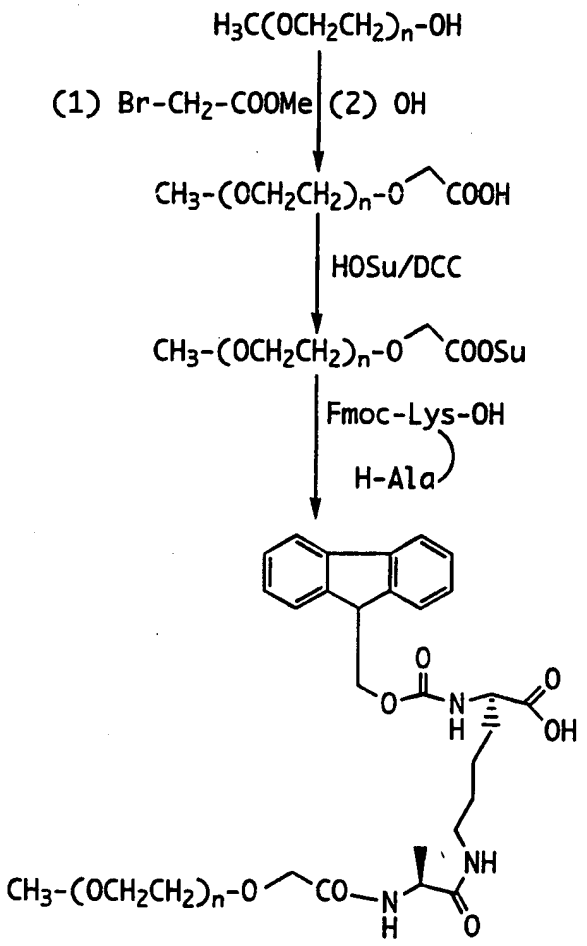

SECONDARY AMINE IN THE BACKBONE OF THE PEPTIDE WILL AFFORD A CHARGED GROUP FOR POTENTIAL INTERACTION WITH PHOSPHATES OF DNA BACKBONE

FIG. 8.

FIG. 9.


H3C(OCH2CH2)n-OH
(1) Br-CH2-COOMe
(2) OH
CH3-(OCH2CH2)n-O
COOH
HOSu/DCC
CH3-(OCH2CH2)n-O
COOSu
Fmoc-Lys-OH
H-Ala
O
O
N
H
O
OH
NH
CH3-(OCH2CH2)n-O
CO
N
H
O

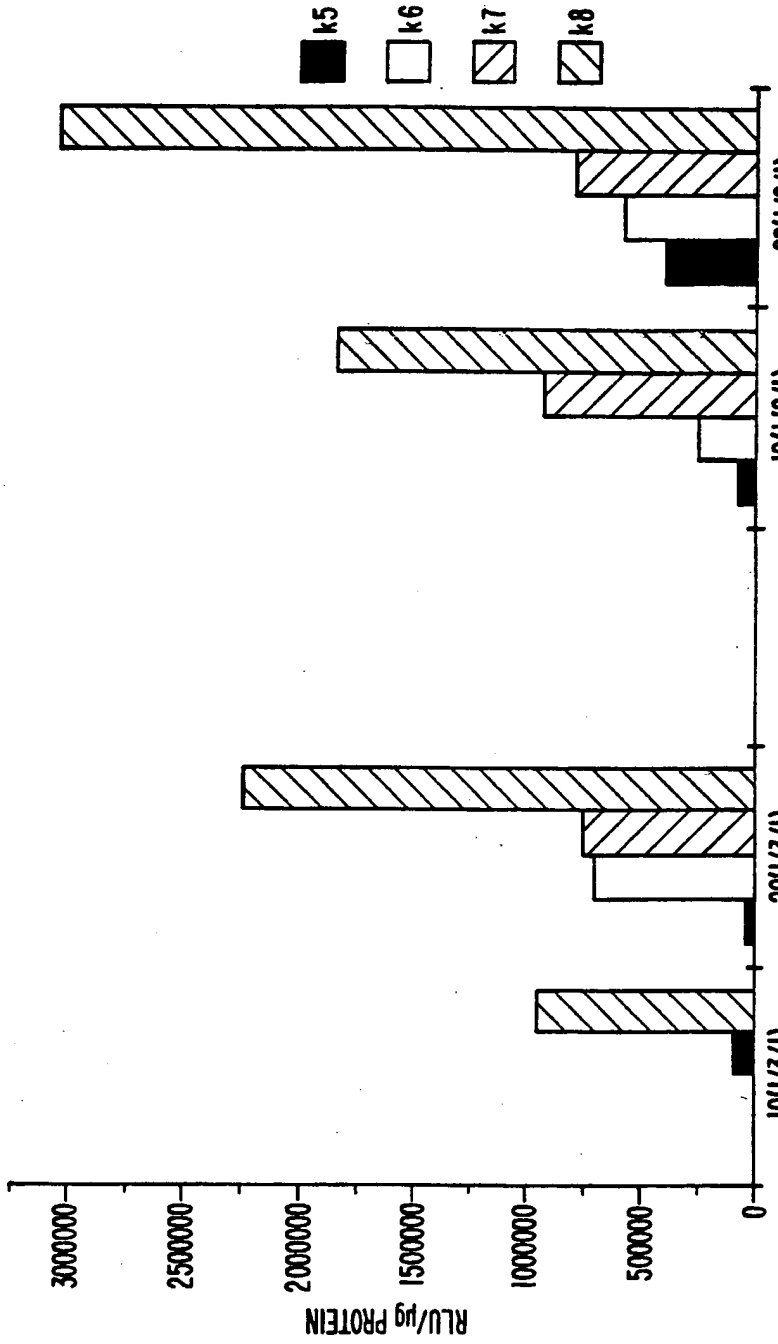

k5
k6
k7
k8
3000000
2500000
2000000
1500000
1000000
500000
0
RLU/µg PROTEIN
10(1/3/1)
20(1/3/1)
10(1/6/1)
20(1/6/1)
COMPLEXES

FIG. 10.

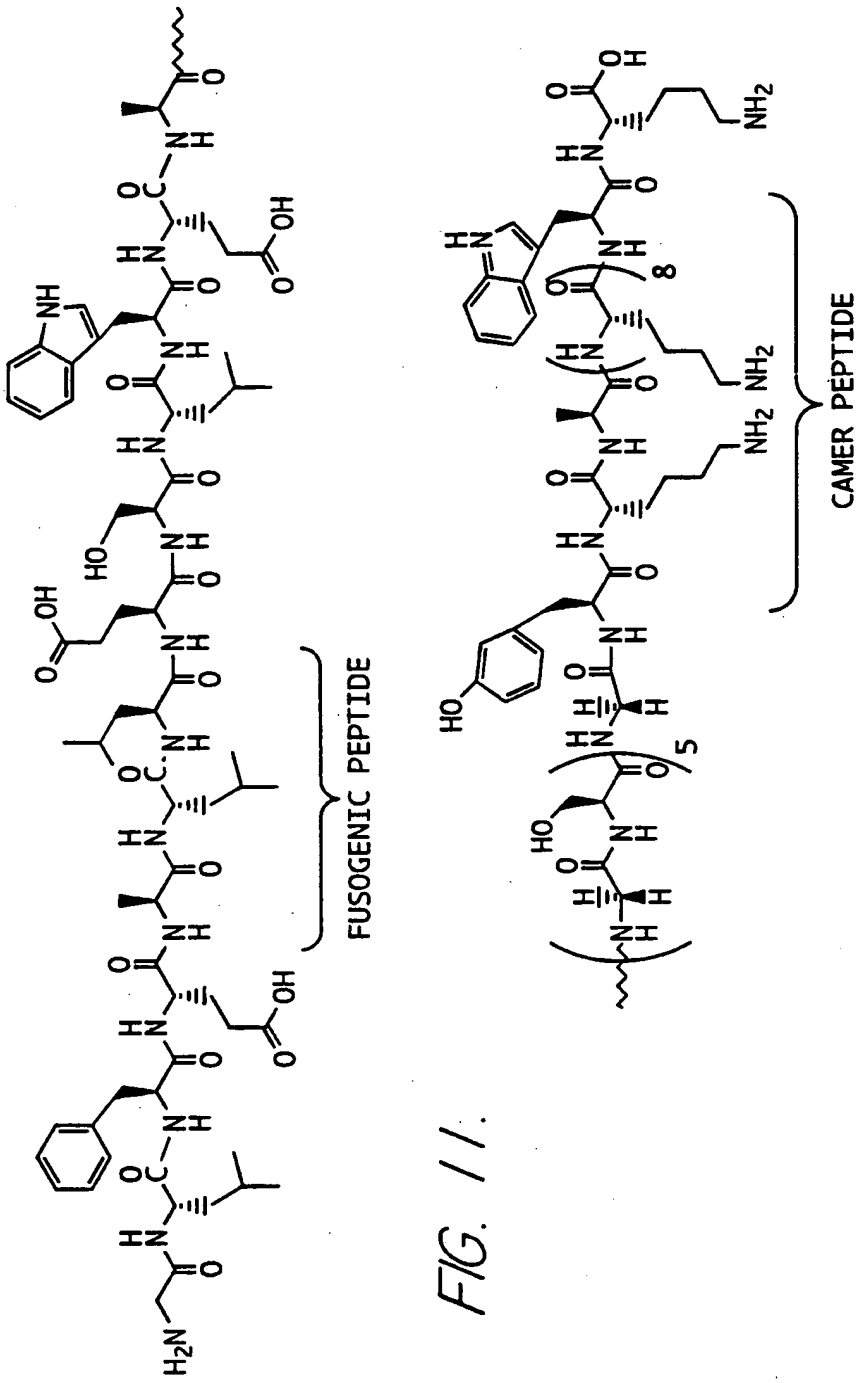


FIG. 11.

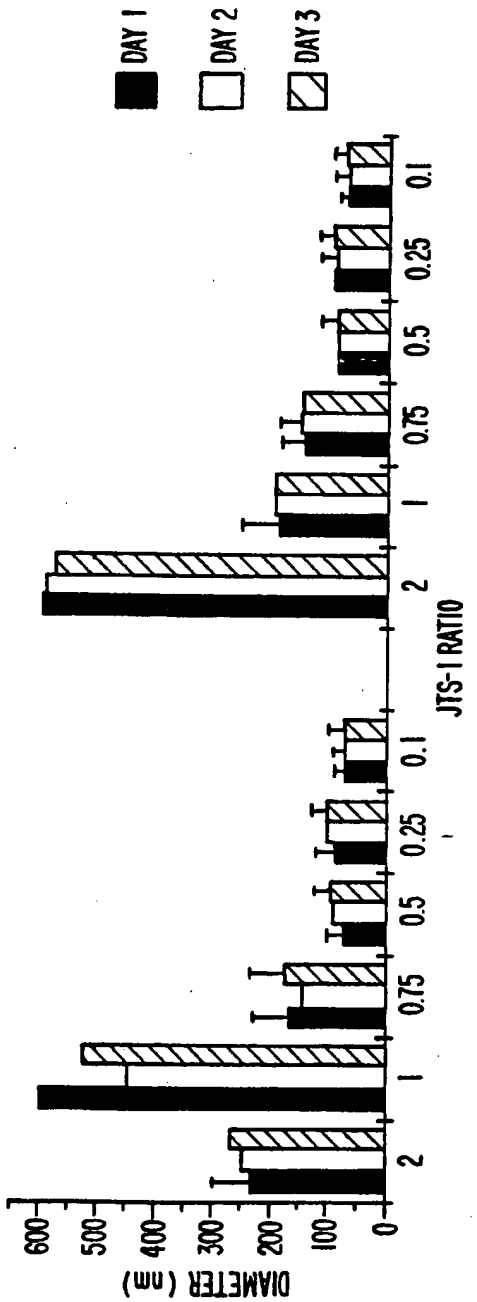


FIG. 12a.

FIG. 12b.

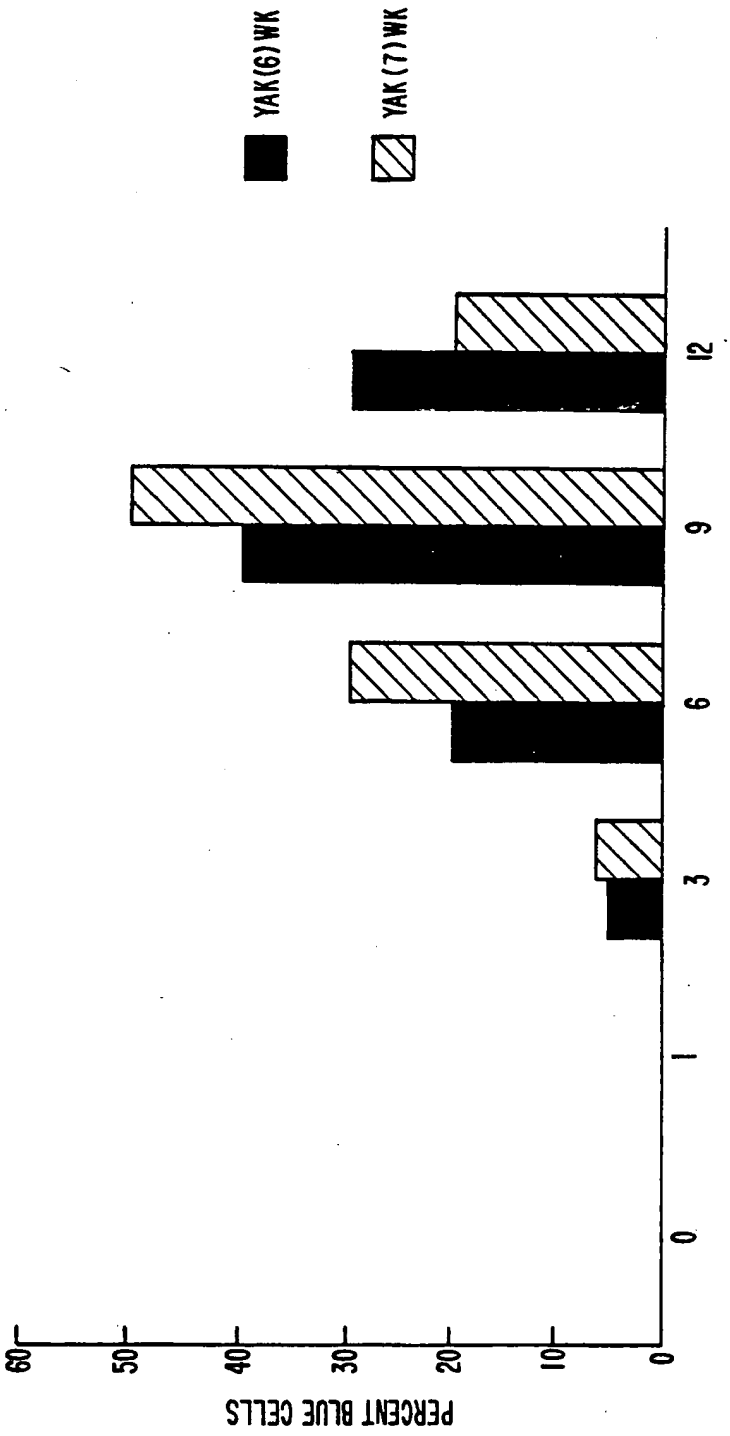

YAK(6) WK
YAK(7) WK
PERCENT BLUE CELLS
60
50
40
30
20
10
0
0
1
3
6
9
12
DNA, µg

FIG. 13.

FIG. 14.

TARGETING LIGANDS

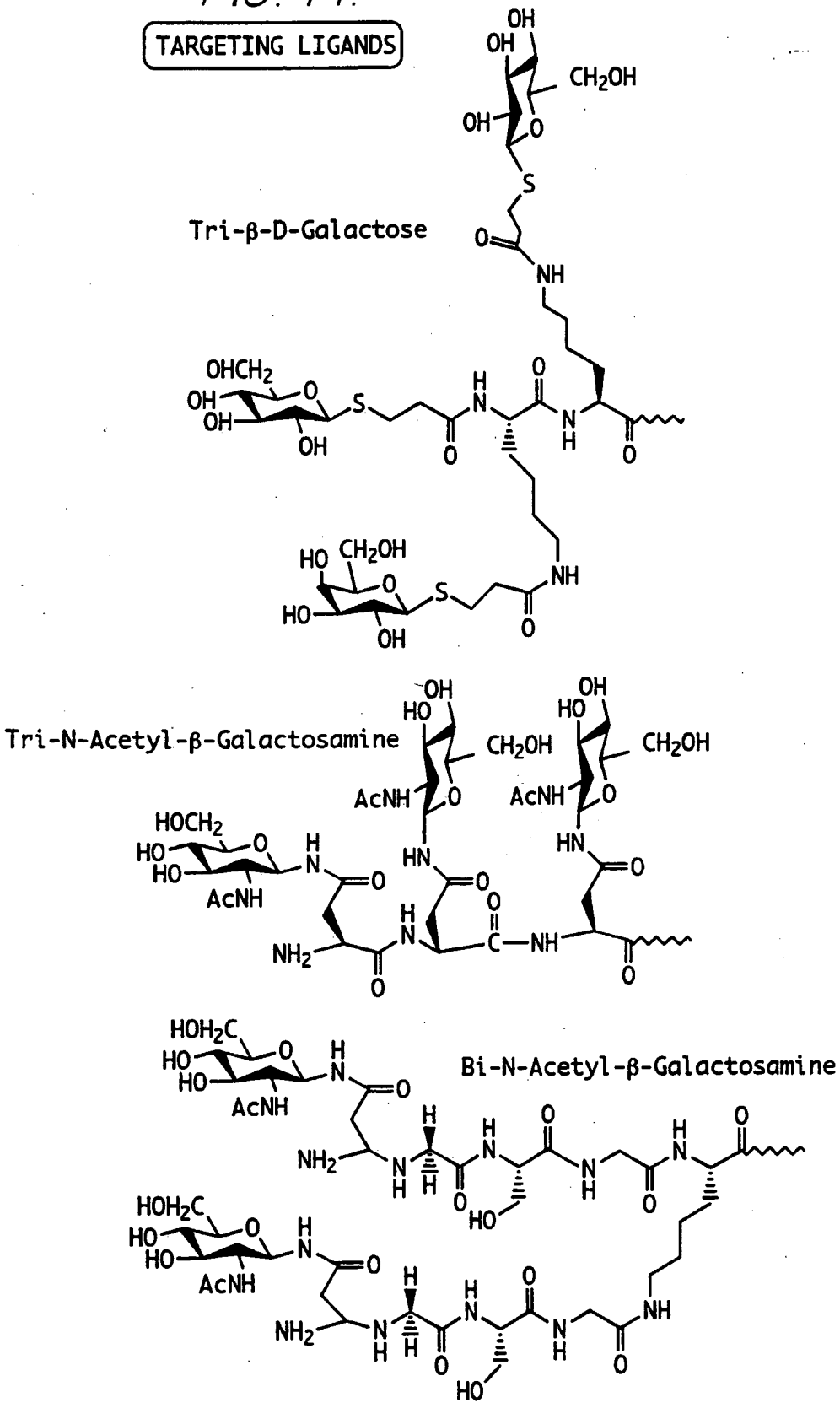
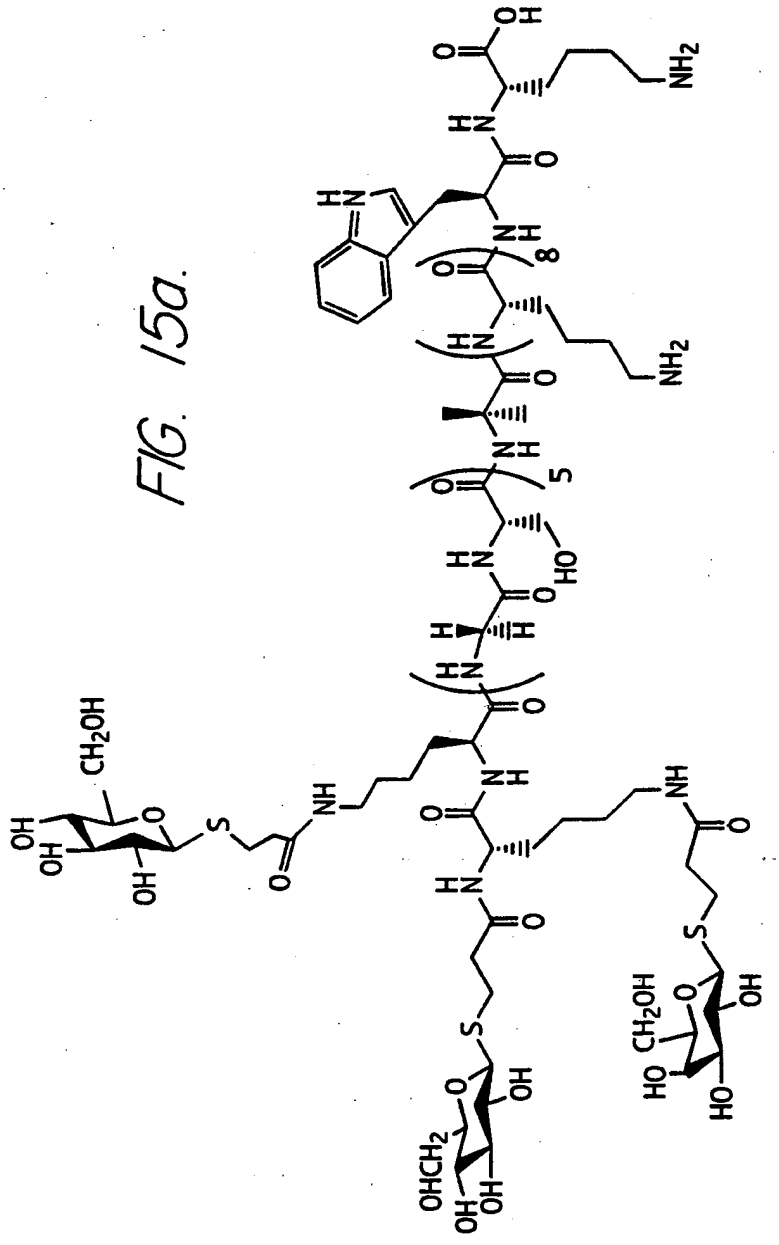
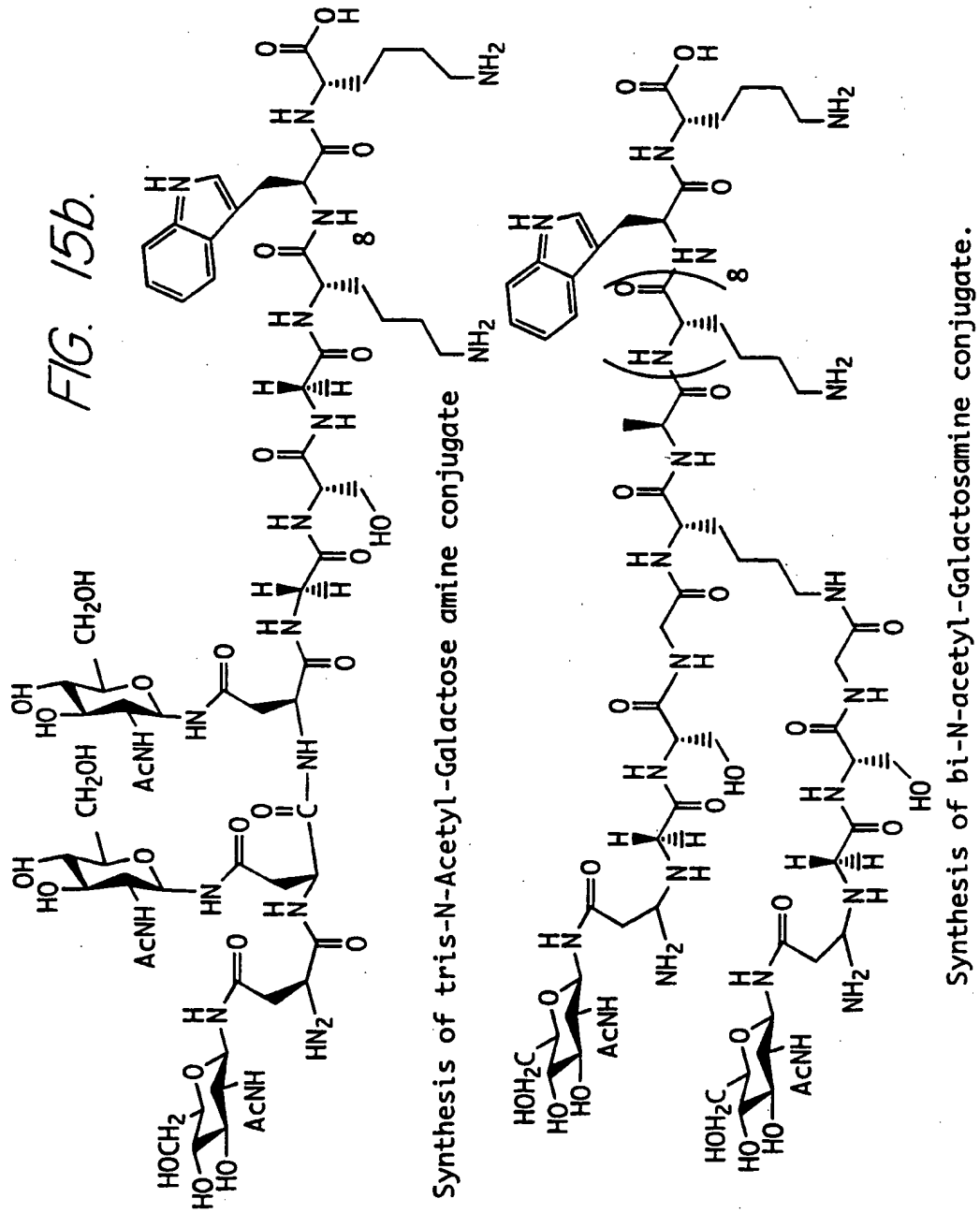


FIG. 15a.



Synthesis of tris-Gal conjugate.

FIG. 15b.



Synthesis of tris-N-Acetyl-Galactose amine conjugate

Synthesis of bi-N-acetyl-Galactosamine conjugate.

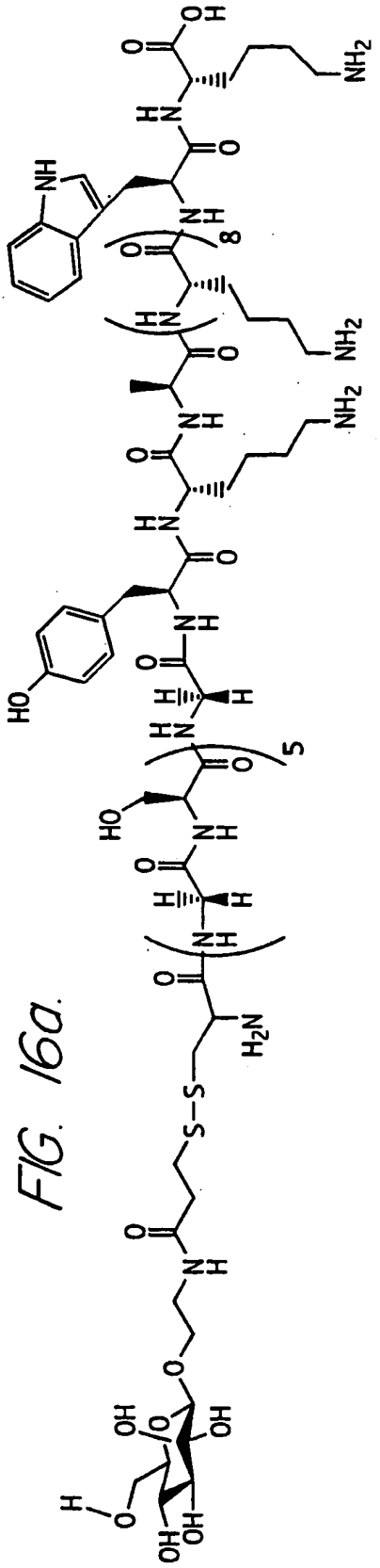
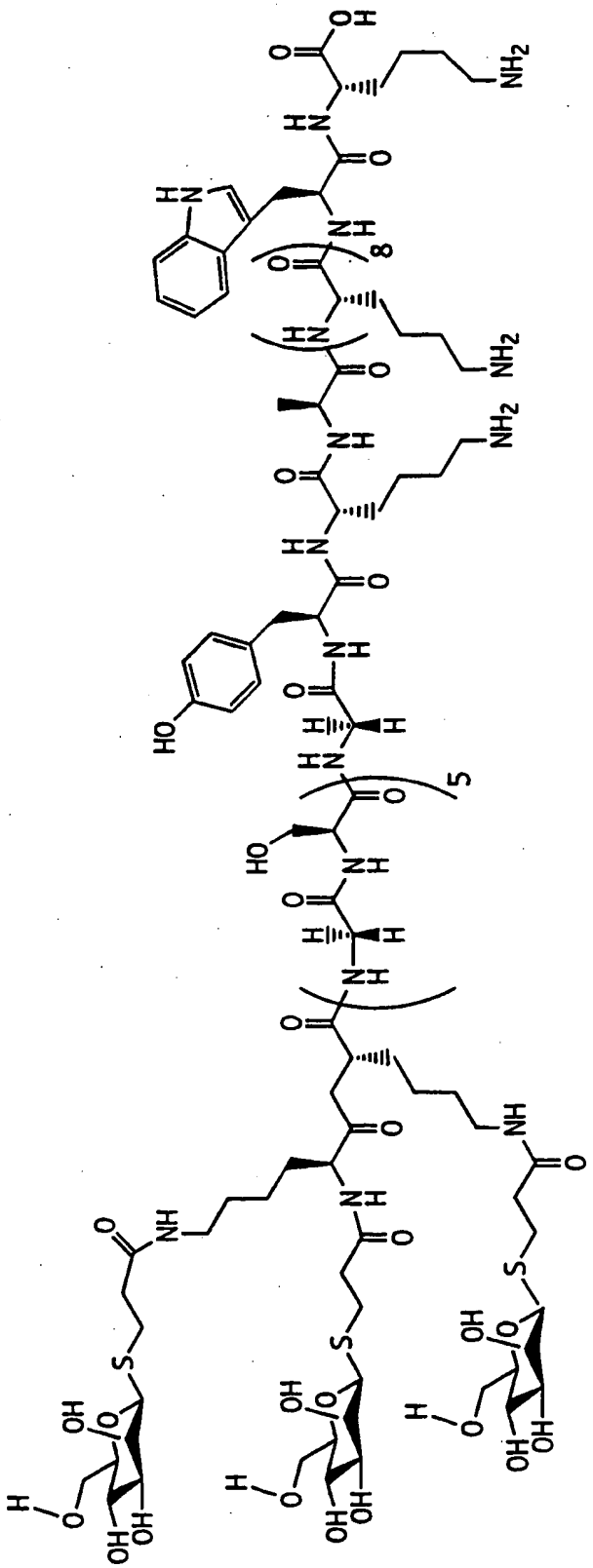


FIG. 16a.



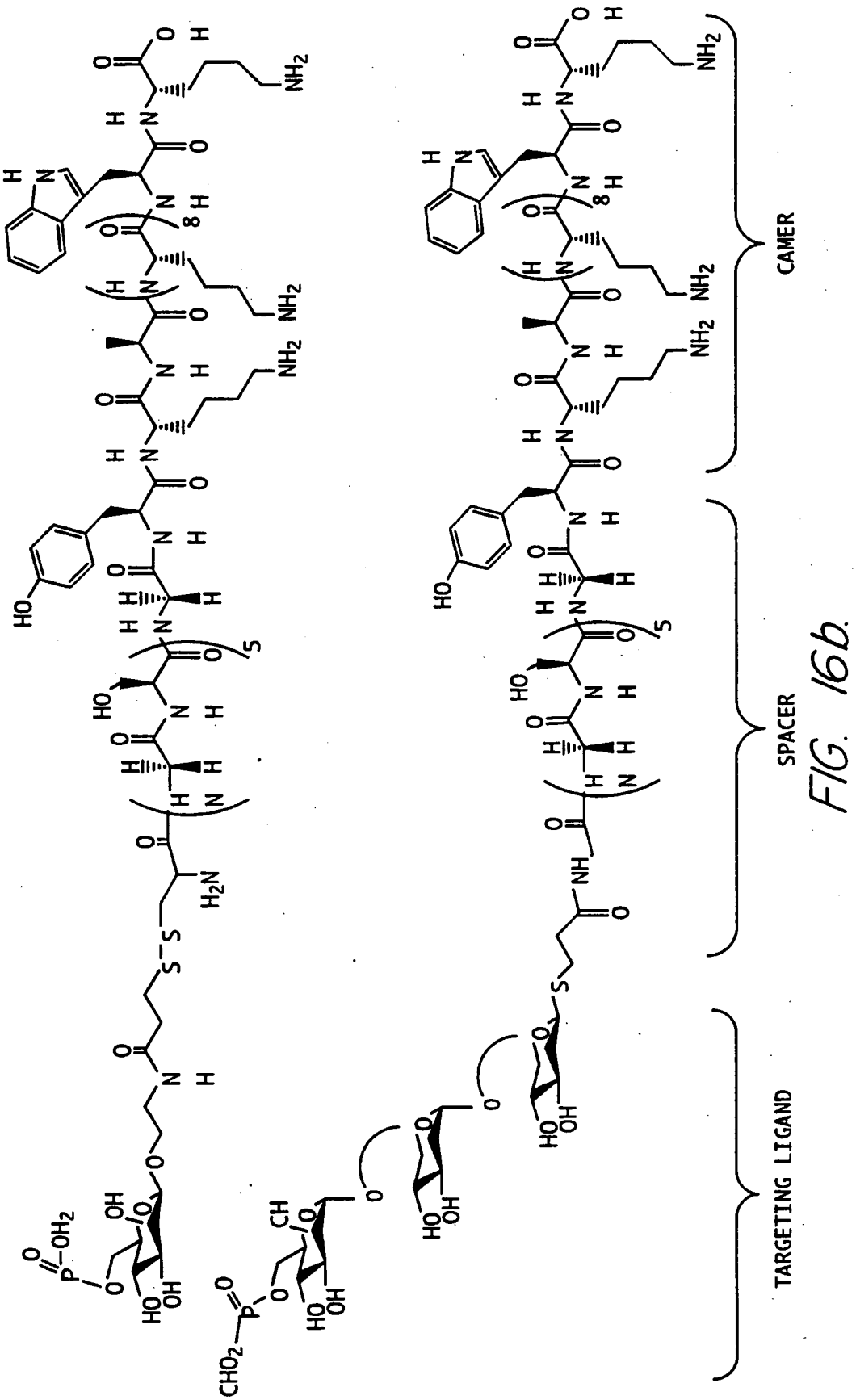


FIG. 16b.

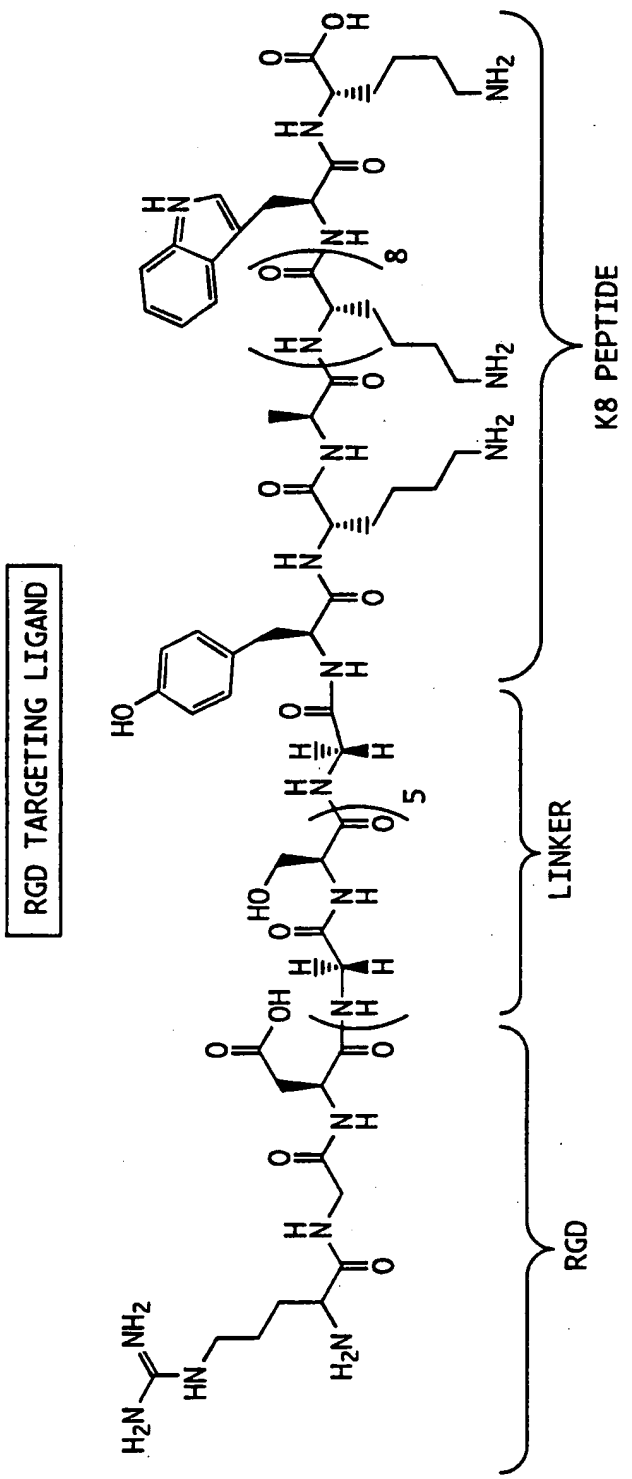
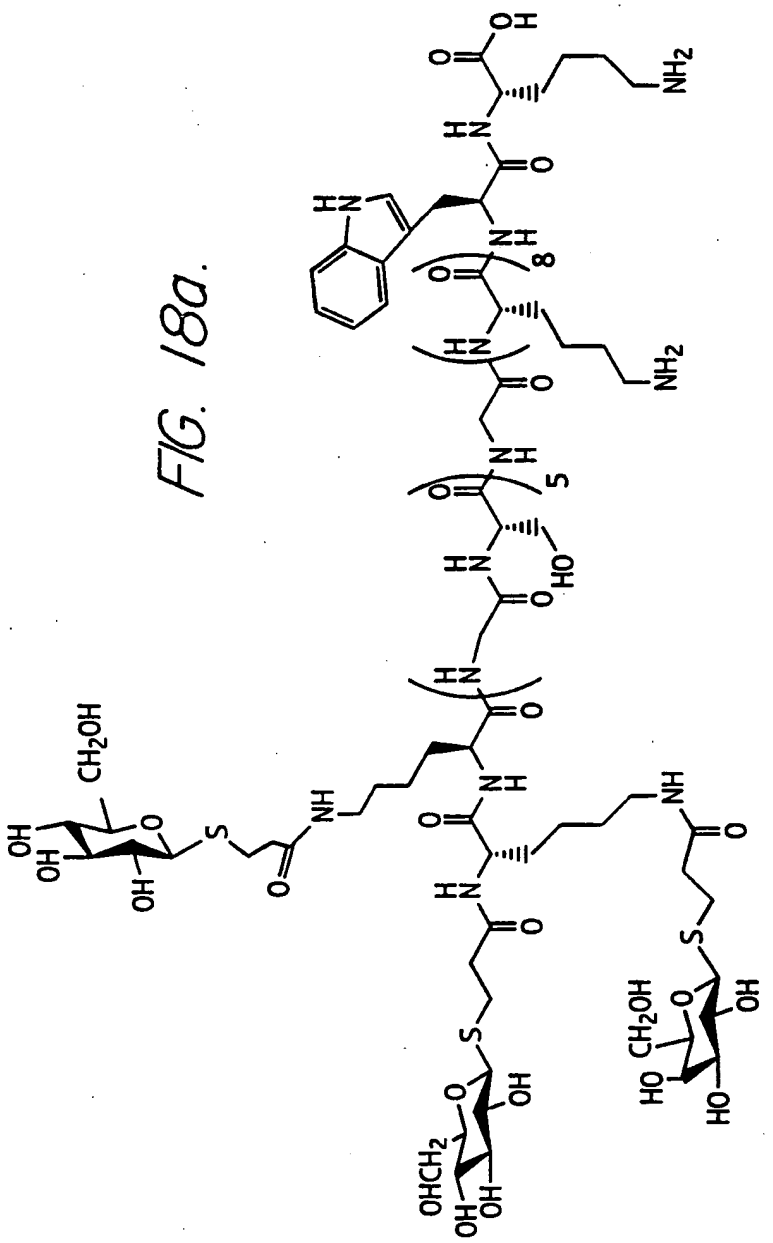


FIG. 17.

FIG. 18a.



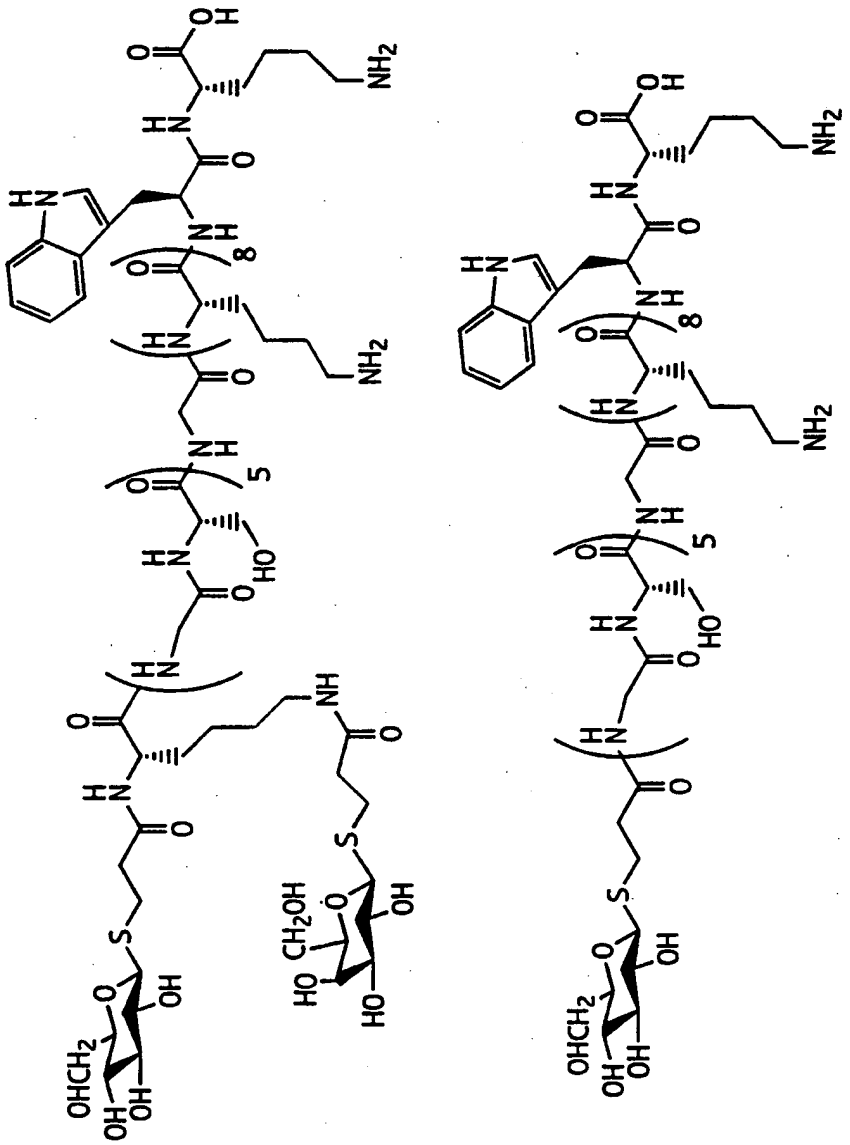


FIG. 18b.

INTERNATIONAL SEARCH REPORT

Interr ial Application No
PCT/US 96/05679

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/87 A61K47/48 A61K48/00 C12N5/10 C07K14/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 18759 (BAYLOR COLLEGE MEDICINE) 30 September 1993	1-3,6,7, 9,10,12, 13, 15-19, 21,22, 24-27,31
Y	see claims 1-48; figures 32,34,36	4,5,11, 14,20, 23,29,30

X	WO,A,93 07283 (BOEHRINGER INGELHEIM INT ;UNIV NORTH CAROLINA (US); GENENTECH INC) 15 April 1993	28
Y	see page 192 - page 193; claims 36,39	4,5,11, 14,20, 23,30
	see claims 1,3,21,31 see page 196	

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 August 1996	07. 08. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/US 96/05679

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 23751 (BOEHRINGER MANNHEIM GMBH ;SUROVOY ANDREJ (DE); DANNULL JENS (DE);) 27 October 1994	28
Y	see page 46; example 1	4,5,11, 14,20, 23,30

X	EP,A,0 359 347 (NEORX CORP) 21 March 1990	28
A	see claims 1-18	4,11,20, 30

Y	CANCER GENE THER.1, 3, 207 1994, XP002009940 E.WAGNER ET AL.: "Receptor-mediated gene transfer : synthetic virus-like systems" see abstract	4,29,30

P,X	GENE THER. (1996), 3(5), 448-457 CODEN: GETHEC;ISSN: 0969-7128, XP002009941 GOTTSCHALK, S. ET AL: "A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells" see the whole document	1-31

2

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US96/05679

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark:Although claim 8 is directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 96/05679

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9318759	30-09-93	AU-B-	3966893	21-10-93
		CA-A-	2131620	30-09-93
		EP-A-	0632722	11-01-95
		JP-T-	7505283	15-06-95
WO-A-9307283	15-04-93	AU-B-	2652692	03-05-93
		BG-A-	98718	28-02-95
		BR-A-	9206559	08-11-94
		CA-A-	2118816	31-03-93
		CZ-A-	9400746	17-05-95
		EP-A-	0545016	09-06-93
		EP-A-	0607206	27-07-94
		FI-A-	941474	30-03-94
		HU-A-	71312	28-11-95
		NO-A-	941154	29-03-94
		NZ-A-	244306	26-07-95
		SK-A-	36894	10-08-94
		ZA-A-	9207460	21-02-94
		CN-A-	1070946	14-04-93
WO-A-9423751	27-10-94	AU-B-	6568594	08-11-94
		DE-A-	4412629	26-01-95
		EP-A-	0693939	31-01-96
EP-A-0359347	21-03-90	US-A-	5135736	04-08-92
		CA-A-	1334513	21-02-95
		JP-A-	2124833	14-05-90
		US-A-	5169933	08-12-92